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(54) Title: METHODS OF USING AGMATINE TO REDUCE INTRACELLULAR POLYAMINE LEVELS AND TO INHIBIT INDUCIBLE NITRIC OXIDE SYNTHASE

(57) Abstract

The present invention provides a method of reducing polyamine levels intracellularly by administering an arginine derivative to a mammal. The present invention also provides a pharmacological composition comprising agmatine in a physiologically acceptable buffer. Accordingly, the present invention also provides a method of treating conditions resulting from abnormally elevated intracellular polyamine levels by administering an arginine derivative or agmatine to the cells in condition such as cancer or hypertrophy. The present invention further provides a method of regulating inducible nitric oxide synthase while maintaining constitutive nitric oxide synthase, by administering agmatine or an arginine derivative to a mammal. The present invention further provides a method of treating septic shock in a mammal, by administering a composition comprising agmatine or an arginine derivative to a mammal. In addition, the present invention provides a method of treating conditions resulting from excessive inducible nitric oxide production, including treatment of septic shock, arthritis, glomerulonephritis, angiogenesis in tumors, transplantation and tissue graft rejection, neurodegeneration, stroke, ischemic injury, chronic inflammation and diabetes.

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METHODS OF USING AGMATINE TO REDUCE INTRACELLULAR POLYAMINE LEVELS AND TO INHIBIT INDUCIBLE NITRIC OXIDE SYNTHASE

BACKGROUND OF THE INVENTION

This invention relates generally to the fields of biochemistry and medicine, and more specifically to controlling polyamine levels and inhibiting inducible nitric oxide synthase in cells and tissues.

Abnormal cellular polyamine biosynthesis has been thought to be related to abnormal cell growth such as cancer, cell enlargement and hypertrophy. The enzyme ornithine decarboxylase (ODC) is rate-limiting in cellular polyamine biosynthesis. ODC is transiently induced in cells treated with growth factors, through activation of tyrosine kinase receptors, immediate early genes and other factors.

Over-expression of ODC in cells leads to cellular transformation. The ODC enzyme is constitutively activated in cells transformed by oncogenes, carcinogens or viruses. Preventing polyamine synthesis in mammalian cells through the use of inhibitors can result in complete cessation of growth. The search for inhibitors that block polyamine biosynthesis for use as therapeutic agents is an ongoing endeavor. Some compounds have been effective in blocking polyamine biosynthesis, but toxic effects of these compounds on cells or organisms occur.

Prior to the present disclosure, the only known way to inhibit a cell from producing polyamines was by administering polyamines to the cell. However, when the goal is to decrease the accumulation of polyamines within a tissue as a treatment for a pathological condition such as a tumor growth, for example, it is important to find

alternatives to the administration of a polyamine that may have a stimulatory effect on cell metabolism.

Compounds that inhibit the enzyme ODC also can inhibit another enzyme, the nitric oxide synthase enzyme (NOS). Toxic cellular effects are observed when nitric oxide (NO) is generated by cells. NO is produced by the conversion of the amino acid L-arginine to L-citrulline by NOS.

While NO can confer cellular antimicrobial activity, this protection can also result in inflammatory damage to host cells and tissues by the potential injurious nature of high NO levels. Septic shock, for example, is the leading cause of death in intensive care units and occurs when microbial products trigger systemic inflammatory responses. The resultant induction of inflammatory cytokines causes a dramatic, irrepressible fall in central blood pressure and, eventually, organ failure. NO is the major contributor to this non-responsive vasodilation, as high NO levels are known to increase during infection, and NOS inhibitors can reverse hypotension and increase survival. However, in other studies such drugs worsened the outcome. NO also is elevated in autoimmune disease such as glomerulonephritis and arthritis, indicating that NO production may be important in the pathogenesis of autoimmune disease. Abnormalities in tumor vasculature may also be attributed to increased NO. NO production has also been associated with increased vascularization in nude mice, resulting in rapid progression of tumor growth. It is advantageous, therefore, to selectively modify NOS activity without altering NO levels required for normal homeostasis.

Thus, a need exists to identify compositions useful for reducing intracellular polyamine levels and inhibiting deleterious effects of NO accumulation in

cells, in order to ameliorate or prevent various cellular pathologies. The present invention satisfies this need and provides additional benefits as well.

SUMMARY OF THE INVENTION

The present invention provides methods of using an arginine derivative to reduce intracellular polyamine levels and to inhibit inducible NOS activity. In one embodiment, the invention provides methods of reducing polyamine levels intracellularly by administering a composition, comprising an arginine derivative such as the compound agmatine. As disclosed herein, agmatine inhibits the enzyme ODC and represses polyamine uptake into cells. The invention also provides a pharmacological composition, comprising agmatine in a physiologically acceptable buffer, that can be administered to a subject in order to reduce intracellular polyamines. The invention further provides methods of inhibiting hyperplasias such as kidney hypertrophy, liver and smooth muscle hyperplasia and the growth of tumor cells by administering agmatine to the affected cells.

In a second embodiment, the invention provides methods of selectively inhibiting inducible nitric oxide synthase (iNOS), while maintaining constitutive nitric oxide synthase (cNOS), by administering an arginine derivative. An arginine derivative can be, for example, agmatine or an agmatine metabolite, agmatine-aldehyde (guanidinobutyraldehyde). The invention also provides methods of treating endotoxic shock in a mammal by administering a composition, comprising agmatine to the mammal. The invention further provides methods of treating conditions resulting from excessive NO generation, including arthritis, glomerulonephritis, angiogenesis in tumors, transplantation and tissue graft

rejection, neurodegeneration, stroke, ischemic injury, chronic inflammation and diabetes, by administering an arginine derivative to an individual suffering from the condition.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 shows the increase in ODC activity in tubules at 24 hours post-nephrectomy, compared with the suppression of arginine decarboxylase (ADC) activity in tubules at 24 hours post-nephrectomy.

Figure 2 shows ODC activity of tubules post-nephrectomy when incubated for 1 hour with buffer, with
1 mM agmatine (+ Agmatine) or 1 mM putrescine (+ Putrescine) compared with the controls.

Figure 3 shows ODC activity of immortalized proximal tubule cells (MCT). Figure 3A shows ODC activity when the cells were incubated for 16 hours in the presence of varying concentrations of agmatine. Figure 3B shows ODC activity of MCT cells incubated for 16 hours without inhibitors, or in the presence of 10 mM eflornithine (DFMO), 1 mM agmatine, or 1 mM putrescine.

Figure 4 shows the change in ODC activity in MCT cells, comparing control cells with cells exposed to 1 mM agmatine for the times indicated.

Figure 5 shows ODC activity in MCT cells in the presence of various inhibitors. Figure 5A shows ODC activity of control cells compared to cells incubated with agmatine in the presence or absence of cycloheximide (CHX). Figure 5B compares ODC activity of control cells to cells incubated with agmatine in the presence or absence of actinomycin-D.

Figure 6 shows the effect of inhibitors and agmatine on ^{3}H-agmatine uptake into MCT cells. Figure 6A shows inhibition by the polyamines putrescine, spermidine, and spermine, the polyamine transport inhibitor paraquat, guanidinobutyric acid (GBA), ornithine, lysine, arginine, and the arginine cationic transporter inhibitor N^5-monomethyl-L-arginine (L-NMMA). Figure 6B shows the effect of preincubation of agmatine (1 mM) on ^{3}H-agmatine uptake in MCT cells over time.

Figure 7 shows the effect of DNA synthesis in MCT cells as indicated by ^{4}H-thymidine incorporation at 48 hours after addition of 1 mM agmatine or 1 mM agmatine plus 50 μM putrescine.

Figure 8 shows the effect of increasing concentrations of agmatine (10 μM to 1 mM), putrescine, spermidine, paraquat, ornithine, lysine and arginine on ^{4}H-putrescine uptake in MCT cells.

Figure 9 shows the effect of preincubation of agmatine (1 mM) on ^{3}H-putrescine uptake in MCT cells over time (0 to 24 hours).

Figure 10 shows the effect of agmatine in the presence of actinomycin-D or cycloheximide (CHX) on ^{3}H-putrescine uptake in MCT cells.

Figure 11 shows agmatine inhibition of both polyamine transport (TSP) and ODC activity in the presence of actinomycin-D or CHX.

Figure 12 shows ODC inhibition using extracts from MCT cells treated with increasing amounts of agmatine.

Figure 13 shows ODC inhibition in the presence or absence of anti-antizyme IgG (anti-AZ) or an antizyme inhibitor (Ain) using extracts from MCT cells treated with 10 mM agmatine.

Figure 14 shows the effect of agmatine, spermine, spermidine, putrescine, GBA or ornithine on the generation of cytokine-induced nitric oxide end products in MCT cells.

Figure 15 shows the effect of agmatine (1 mM) on the generation of cytokine-induced NO end products in MCT cells over time.

Figure 16 shows the effect of diamine oxidase (DAO) on the generation of cytokine-induced NO end products in MCT cells. Figure 16A shows the effect of increasing concentrations of DAO. Figure 16B shows the effect of increasing concentrations of DAO and 1 mM agmatine.

Figure 17 shows the effect of the DAO inhibitor, pentamidine, and agmatine (1 mM) on the cytokine-induced NO end products in MCT cells.

Figure 18 shows the effect of pentamidine with increasing concentrations of agmatine on cytokine-induced NO end products in MCT cells.

Figure 19 shows the effect of aldehyde dehydrogenase (AldDH) on the generation of cytokine-induced NO end products in MCT cells. Figure 19A shows the effect of increasing amounts of AldDH. Figure 19B shows the effect of increasing amounts of AldDH and agmatine (1 mM). The effect of the AldDH cofactor, nicotinamide adenine dinucleotide (NAD), is also shown.

Figure 20 shows the effect of AldDH and increasing amounts of agmatine on the generation of cytokine-induced NO end products in MCT cells.

Figure 21 shows the inhibition of NO end product accumulation as a result of increasing amounts of agmatine in various cell lines.

Figure 22 shows the effect of administering lipopolysaccharide (LPS) and agmatine plus LPS in Wistar Fromter rats. Figure 22A shows the change in blood pressure. Figure 22B shows the change in glomerular filtration rate (GFR).

DETAILED DESCRIPTION OF THE INVENTION

The present invention provides a method of reducing polyamine levels intracellularly by administering the compound agmatine to cells or tissues of a mammal. As disclosed herein, agmatine, which is an arginine derivative, can inhibit the enzyme ornithine decarboxylase (ODC), reduce polyamine uptake into cells, and inhibit the inducible nitric oxide synthase (iNOS) enzyme while maintaining constitutive nitric oxide synthase (cNOS) enzyme levels in cells.

As used herein the term "polyamine" refers to the naturally occurring polyamines spermidine, spermine, as well as the diamine precursor putrescine. The intracellular concentrations of polyamines affect cell growth and development. Putrescine is derived from ornithine through the action of ODC. Subsequently, spermidine is formed from putrescine via the enzyme spermidine synthase in conjunction with a decarboxylated-adenosylmethionine, and spermine is formed from spermidine and decarboxylated-adenosylmethionine using the enzyme spermine synthase.

The diamine putrescine ($H_2N(CH_2)_4NH_2$) and the polyamines spermidine ($H_2N(CH_2)_3NH(CH_2)_4NH_2$) and spermine ($H_2N(CH_2)_3NH(CH_2)_4NH(CH_2)_3NH_2$) are present in all mammalian cells and are involved in the initiation and progression of the cell cycle, as well as cell hypertrophy and differentiation. The biosynthesis of polyamines precedes both protein and nucleic acid synthesis in the cell cycle. ODC catalyzes the decarboxylation of ornithine to produce putrescine, from which spermidine and spermine are produced.

Agmatine is a polyamine, specifically, a decarboxylated arginine derivative having the chemical name guanidinobutylamine or 4-(aminobutyl)guanidine ($C_5H_{14}N_4$). Agmatine has a molecular weight of 130.19. Arginine is converted to agmatine by arginine decarboxylase (ADC). Arginine is critical to normal cellular growth and multiple physiological processes. Arginine concentration in extracellular fluid is maintained at 100 to 200 μM and is regulated by gastrointestinal absorption, conversion to ornithine by the urea cycle, and synthesis from citrulline in the kidney (Lortie et al., J. Clin. Invest. 97:413-420 (1996)). The metabolites of arginine include NO, which is generated from arginine by NOS, and agmatine, which is a decarboxylated arginine derivative produced from arginine by ADC.

Derivatives of agmatine inhibit NOS activity as shown, for example, in the assays described in the Examples below. Derivatives that increase the half-life of the agmatine molecule by decreasing susceptibility to diamine oxidase (DAO) are one type of derivative. Since the agmatine molecule is a four carbon chain separating two positively charged groups, agmatine derivatives useful in the invention maintain the four carbon chain with two positively charged groups at either end.

However, the amine group at one end can be modified, for example, by methylation of the amine or by substitution of a second guanidinium group in place of the amine, or by other types of modification that would prevent oxidation of the amine to the aldehyde by DAO, thus producing an agmatine derivative. Agmatine can be used as a pharmaceutical composition, for example, a salt formulation, or a zwitterionic form of the molecule, or can be formulated in a composition or conjugated to a carrier molecule.

Agmatine-aldehyde (guanidinobutyraldehyde) is a particular agmatine derivative in which the amine group of agmatine is oxidized to an aldehyde. Agmatine is converted by DAO to agmatine-aldehyde, which is unstable and is further metabolized to GBA by aldehyde dehydrogenase (AldDH).

As disclosed herein, arginine derivatives, in addition to agmatine, are useful in practicing the methods of the invention. Arginine derivatives are metabolites of arginine that have a demonstrated ability to reduce NO end product accumulation or biological activity as determined using the assays described in the Examples, or other similar assays known to those in the art. Arginine derivatives generally are products of the arginine decarboxylase pathway, including agmatine and agmatine-aldehyde, but also can be synthetic arginine derivatives.

The synthesis of agmatine is described by Kosel (Physiol. Chem. 68:170 (1910)), which is incorporated herein by reference. Agmatine is available from commercial vendors such as Sigma Chemical Company (St. Louis, MO). Agmatine is a naturally occurring endogenous molecule that concentrates in some organs and is also a component of plasma, which allows distribution

of agmatine to all tissues. Agmatine enters cells by the polyamine transport system and its uptake can be competitively inhibited using polyamines or polyamine transport inhibitors (see Figure 6 and Example I).

ODC is the rate limiting enzyme of polyamine biosynthesis and is one of the most highly regulated eukaryotic enzymes. ODC, which is the convergence point of many oncogenic signaling pathways, is a proto-oncogene, whose over-expression leads to transformation of certain cells. Therefore, polyamines are also thought to play a proximate role in the transformation process. ODC exhibits the shortest half-life of any described enzyme and is transiently induced in cells in response to various conditions, including following growth factor addition, activation of tyrosine kinase receptors, in hypoxia, following cellular free radical formation, through prostaglandin activity and by immediate early gene activity. In contrast, ODC is constitutively active in cells transformed by oncogenes, carcinogens or viruses.

Although ODC is related to the process of cellular transformation, modulating this enzyme with inhibitors such as eflornithine (DFMO) does not prevent proliferation or hypertrophy of cells in experimental models reported to date. The levels of spermidine and spermine inside cells were largely unaffected by inhibition of ODC and these levels were sufficient to allow the normal progression of events in models of kidney hypertrophy, liver hyperplasia and smooth muscle hyperplasia. Cells that are unable to synthesize polyamines, for example, due to the presence of the inhibitor DFMO, are still capable of taking up polyamines from the environment.

Polyamines regulate their biosynthesis by feedback inhibition, indirectly inducing the translational expression of the protein ODC antizyme (AZ). AZ has a dual function, inhibiting both ODC activity and polyamine transport into the cell.

Abnormal polyamine biosynthesis is associated with abnormal cell growth such as cancer, cell enlargement and hypertrophy. Preventing polyamine synthesis in mammalian cells through the use of inhibitors results in complete cessation of growth unless exogenous polyamines are provided (Pegg, Cancer Res. 48:759-774 (1988)). The use of inhibitors that block polyamine biosynthesis as therapeutic agents is directed towards a variety of diseases involving pathological cell proliferation or cell enlargement.

Synthetic ODC inhibitors have been tested for therapeutic impact on abnormal cell proliferation and cell enlargement. Presently known inhibitors of ODC can be classified as reversible inhibitors, such as direct competitors, and enzyme-activated irreversible inhibitors. The latter, often referred to as "suicide" inhibitors, are chemically inert substrates for the enzyme, that inactivate the enzyme upon binding. The most commonly used inhibitor is DFMO (Pegg, supra, 1988).

However, the use of ODC inhibitors does not always produce the intended results. For example, in models of smooth muscle hyperplasia in the rat (Luck et al., Am. J. Physiol. 267:G1021-G1027 (1994)), as well as in a model of liver regeneration (Beyer et al., Am. J. Physiol. 262:G677-G684 (1992)), the administration of the ODC inhibitor DFMO did not prevent hyperplasia or the regeneration of liver cells. Putrescine content in the tissues was reduced after the administration of DFMO, but the level of spermine and spermidine was unchanged.

Reducing the biosynthesis of polyamines intracellularly increased uptake of polyamines from the surrounding tissue.

Polyamines, in normal cell types, are the products of a highly regulated intracellular biosynthetic pathway. Polyamines are transported into and out of cells through temperature sensitive, energy dependent transporters (Humphries et al., Am. J. Physiol. 255:F270-F277 (1988)). Polyamine uptake can substitute for de novo synthesis. Extracellular polyamine uptake can be enhanced by many of the same factors that induce ODC activity, for example, growth factors and hormones. Polyamine transport is inhibited by the induction of intracellular biosynthesis and, conversely, biosynthesis is inhibited by the induction of transport, thereby demonstrating a system highly sensitive to intracellular polyamine levels. Although extracellular polyamine levels generally are far lower than intracellular concentrations, plasma polyamine levels, as well as cellular uptake, are often markedly elevated in malignancy. Therefore, inhibitors targeting only polyamine biosynthesis have had very limited success as cancer therapeutics.

AZ inhibits polyamine uptake and inhibits and destabilizes ODC. The degradation of ODC is catalyzed by 26S protease (Murakami et al., Nature 360:597-599 (1992)). ODC is destabilized by AZ, where the C-terminal half of AZ binds to ODC, inducing a conformational change (Li et al., Mol. Cell. Biol. 13:2377-2383 (1993)) allowing an additional internal sequence in AZ to promote destabilization of ODC (Li et al., Mol. Cell. Biol. 14:87-92 (1994); Ichiba et al., Biochem. Biophys. Res. Commun. 200:1721-1727 (1994)). As a result, ODC is not only short-lived like other key proteins, but its turnover is regulated. The induced destabilization by AZ

is analogous to the human papilloma virus oncoprotein E6 action on the tumor suppressor p53 (Scheffner et al., Cell 53:1129-1136 (1990); Matsufuji et al., Cell 80:51-60 (1995)).

AZ has a second function, repressing polyamine uptake, thereby effectively preventing polyamine accumulation in cells. Researchers using ODC-overproducing cells transfected with an AZ cDNA found a decrease in polyamine transport into the cells and subsequent cellular toxicity when an ODC inhibitor was administered, compared with controls where polyamine transport increased in response to the use of the ODC inhibitor DFMO (Suzuki et al., Proc. Natl. Acad. Sci., USA 91:8930-8934 (1994)). Therefore, induction of AZ inhibits polyamine biosynthesis as well as transport. However, until the present invention, only the natural polyamines, putrescine, spermidine and spermine were known to induce AZ in a feedback regulatory manner.

AZ induction by polyamines occurs at the translational level. AZ synthesis was blocked by cycloheximide (CHX), but not by actinomycin D (Fong et al., Biochim. Biophys. Acta 428:456-465 (1976); Matsufuji et al., J. Biochem. 107:87-91 (1990)). While very low levels of AZ are present in mammalian tissues, mRNA levels are relatively high and not further elevated by polyamines (Matsufuji et al., supra, 1990). The mechanism of polyamine feedback inhibition through AZ involves modulation of frameshifting due to the cell concentration of polyamines (Matsufuji et al., supra, 1995).

The present invention provides methods of decreasing intracellular polyamine levels by administering an arginine derivative, such as agmatine or a derivative thereof, to cells or tissues of a mammal.

As disclosed herein, administering agmatine to a cell culture or tissue inhibits ODC activity and uptake of polyamines through the induction of AZ. The present invention also provides methods of inhibiting the ODC enzyme by administering a composition, containing an arginine derivative, such as agmatine or a derivative thereof, to the cells or tissues of a mammal.

The inhibitory effect of agmatine on ODC activity is demonstrated in Example I. These experiments use both primary cultures of proximal kidney tubule cells and MCT cell lines. MCT cells, SV-40 transformed proximal tubule cells, like other transformed cell lines, demonstrate high constitutive ODC levels (Olanrewaju et al., Am. J. Physiol. 63(2Pt1):E282-E286 (1992) which is incorporated herein by reference). The transformed cells mimic the cellular hypertrophy (Luck et al., supra, 1994) or the profile of ODC expression in diabetes (Levine et al., Diabetes 29:532-535 (1980)).

Tubule cells from nephrectomized animals have marked inverse changes in the ODC and ADC activities from control tubule cells. ODC activity is elevated 24 hours post-nephrectomy, while ADC activity is decreased. ADC catalyzes the conversion of arginine to agmatine, and its activity is considered indicative of agmatine production in various tissues (Lortie et al., supra, 1996). Agmatine effectively suppresses ODC activity in the tubules. At 1 mM agmatine concentration, ODC activity was almost completely suppressed (Figure 3). The inhibition was reduced by CHX, but not by actinomycin-D (Figure 5), indicating that agmatine inhibition requires translation, but not transcription, to be effective. Further, ODC activity was inhibited by 1 mM agmatine in several cell lines. Agmatine uptake into cells was inhibited by polyamines, and by the polyamine transport inhibitor paraquat, but not by other types of inhibitors,

indicating that agmatine is taken into cells using the polyamine transport system but is not universally transported by cationic transporters (Figure 6). DNA synthesis also was reduced by agmatine (Figure 7). The addition of putrescine bypassed the inhibitory effect of agmatine and demonstrated the specificity of the inhibition. These results demonstrate that a non-feedback dependent pathway exists for ODC regulation by agmatine.

Agmatine inhibits polyamine uptake into cells (see Example III). Concentrations of agmatine (10 μM or higher) effectively reduced putrescine uptake into cells. CHX, but not actinomycin-D, affected the inhibition demonstrating that only translation is required for agmatine inhibition of both ODC and polyamine transport. These results are consistent with the induction of AZ by agmatine. The role of AZ was demonstrated directly in Example IV. Agmatine was administered to MCT cells, then extracts of these agmatine-treated cells were used to inhibit ODC. Extracts of agmatine-treated cells decreased ODC activity in a dose-dependent manner. Extracts of MCT cells treated with 10 mM agmatine were added to an ODC assay mixture. The inhibition was reversed by adding anti-AZ-antibody or an AZ inhibitor. These results indicate that the induction of AZ by agmatine is responsible for the inhibition of both ODC activity and polyamine transport. Agmatine induces translational frameshifting in the rabbit reticulocyte lysate assay (Matsufuji et al., supra, 1995).

The present invention further provides a pharmacological composition containing an arginine derivative, such as agmatine or a derivative thereof, and a physiologically acceptable carrier. Agmatine is typically supplied as a sulfate salt and has a neutral pH in water. Agmatine can be used with many types of

buffers and is typically sterile-filtered using a 0.2 μm filter. As used herein, the term "physiologically acceptable carrier" includes any of the standard pharmaceutical carriers, such as phosphate buffered saline solution, water, or emulsions such as an oil/water or water/oil emulsion and various types of wetting agents.

Exogenous agmatine concentrations of up to 5 mM were well tolerated by all cell lines screened by trypan blue (0.4% solution) exclusion. In contrast, polyamines such as spermine were toxic to cells at concentrations greater than 100 μM. For administration to mammals, dosages of between approximately 5 μg/kg body weight to 80 mg/kg body weight are preferred for the pharmacological compositions of the invention. The preferred dosage will vary with the mode of administration. Multiple or intravenous administrations, for example, allow lower dosages than intramuscular or other routes of administration. Very high dosages of agmatine administered to test animals (up to 80 mg/kg body weight) every three hours were well tolerated. A broad range of dosages is available for the treatment of various pathological conditions. The dosage will vary with the condition being treated as well as the method of administration.

A pharmacological composition of the invention can also include other components to enhance the effectiveness or stability of the arginine derivative. For example, DAO inhibitors such as aminoguanidine or pentamidine can be included to inhibit agmatine metabolism and increase agmatine half-life. DAO converts agmatine to guanidinobutyraldehyde. A pharmacological solution can be administered using a number of methods known in the art, for example, intravenously, intraperitoneally, intramuscularly, intranasally, or

subcutaneously. In some cases, the pharmacological composition can be infused directly into the tissue that is targeted.

The present invention also provides methods of treating pathological conditions by administering a composition, comprising an arginine derivative such as agmatine or a derivative thereof. The pathological conditions most suited for treatment using a composition of the invention are characterized, in part, by abnormal cellular proliferation or hypertrophy, for example, tumor development. Compositions containing agmatine, for example, can be administered directly to cells to prevent proliferation of the cells, or can be administered to an individual to prevent angiogenesis associated with tumor development. A composition of the invention can also be administered to prevent clonal cell expansion in the immune response in order to reduce inflammation or to increase tolerance for transplanted organs.

In cellular hypertrophy, some terminally differentiated cell types, such as kidney tubule cells or cardiac muscle cells, increase in size and protein content, but are then blocked from entry into the cell cycle. An arginine derivative such as agmatine is capable of inducing the same biochemical responses to hypertrophy as it does with respect to proliferative conditions. Methods of treating conditions such as cardiac hypertrophy, renal disease progression and the hypertrophy characteristic of diabetic renal disease by administering a composition comprising an arginine derivative such as agmatine or a derivative thereof are provided by the invention. Polyamines have multiple other functions including acting as antioxidants, modulating differentiation and apoptosis, and regulating transport through channels. Therefore, influencing the

levels of deleterious polyamines in the cells can potentially affect all of these conditions.

AZ was believed to be induced only by ODC in a feedback dependent manner. The present invention provides methods of inducing AZ in a non-feedback dependent manner through the administration of an arginine derivative such as agmatine or a derivative thereof. In addition, other naturally occurring or synthetic molecules can be screened for modulation of AZ expression, thereby manipulating both polyamine biosynthesis and transport. Such a screening method would involve, for example, the use of an anti-AZ antibody, which can be a monoclonal or polyclonal antibody specific for the AZ and made as described, for example, in Harlow and Lane, Antibodies: A laboratory manual (Cold Spring Harbor Laboratory Press, 1988), which is incorporated herein by reference. AZ has been isolated and characterized (Hayashi et al., Ornithine Decarboxylase: Biology, Enzymology, and Molecular Genetics (Hayashi, S., ed.) pp. 47-58, Pergamon Press, NY (1989), which is incorporated herein by reference) and monoclonal antibodies against rat liver AZ (Matsufuji et al., supra, 1990, which is incorporated herein by reference) have been produced. Further, using monoclonal antibodies as probes, cDNAs encoding AZs were cloned from various cells. Using the cDNA as a probe, genomic clones were isolated (Miyaszki et al., Gene 113:191-197 (1992); Hayashi et al., Biochem. J. 306:1-10 (1995), each of which is incorporated herein by reference).

Agmatine and other arginine derivatives are polyamines that function as inhibitors of the enzyme ODC. Other enzymes, including iNOS, can also be inhibited by agmatine. Thus, the present invention also provides methods of selectively inhibiting inducible nitric oxide

synthase (iNOS), while maintaining or enhancing constitutive nitric oxide synthase (cNOS) production.

NO is an inorganic free radical. NO is produced in many cell types by the conversion of L-arginine to L-citrulline and NO through NOS. NOS converts L-arginine to N^G-hydroxy-L-arginine, which is then further converted to citrulline and NO. The biological activity of NO is the result of the activation of various enzymes, for example, guanylyl cyclase, and the inhibition of others, for example, aconitase or ribonucleotide reductase; or activation by alternative mechanisms such as damaging nucleic acids. NO is produced by either cNOS or iNOS enzymes.

NO synthases occur as a family of isoenzymes. Two of the cNOS isozymes are constitutively produced (NOS I and NOS III) and iNOS (NOS II) is induced by immunological stimuli such as endotoxin or inflammatory cytokines. cNOS, first described in brain and endothelial cells, is activated by acetylcholine, bradykinin, and other substances, resulting in short-lived production of NO in picomolar amounts. The NO released by constitutive enzymes acts as an important signaling molecule in cardiovascular and nervous systems. The NO released by iNOS in response to cytokines or endotoxin, is generated for long periods and in nanomolar amounts (Nathan et al., Cell 76:915-918 (1994); Ketteler et al., Am. J. Physiol. 36:F197-F207 (1994)). The iNOS is made by macrophages, hepatocytes, vascular smooth muscle cells, mesangial cells, renal tubular cells, and other cell types and has been shown to be cytostatic and cytotoxic for tumor cells and a variety of organisms. Each isoform contains a reductase as well as a heme domain and requires a number of cofactors. The

enzymes are produced by at least three different genes and range in molecular weight from about 130 kDa to 160 kDa.

In the murine immunological system, lipopolysaccharides (LPS), interferon γ, and other cytokines induce the synthesis of NOS in macrophages and related cells (Granger et al., J. Immunol. 146:1294-1302 (1991)). As a consequence, the NO produced arrests the growth of microbes and tumor cells by several mechanisms. However, iNOS protection against microbes also cause inflammatory damage to host cells and tissues by the potential injurious nature of high NO levels. Deleterious effects include the combination with O_2^- or superoxide ion, where NO can damage DNA and induce mutations. The genotoxic potential may be responsible for initiating various genetic disorders including some cancers. The induction of iNOS has been implicated in numerous pathological conditions, including sepsis-related hypotension, disturbances of the hemostatic-thrombotic balance, and local vascular lesions such as atherosclerosis and post angioplasty arterial injury. In particular, NO-induced hypotension leads to cardiovascular complications in septic shock patients as well as during cytokine-based immunotherapy. Studies in iNOS deficient mice have elaborated on the role of NO in septic shock. When iNOS deficient mice were challenged with bacterial endotoxic LPS, they did not suffer from the fall in central arterial blood pressure and subsequent death caused by septic shock when compared with the control wild type mice (MacMicking et al., Cell 81:641-650 (1995)).

Nonspecific NOS inhibitors can alter various autoimmune diseases including glomerulonephritis and arthritis, indicating that elevated NO production is important in the pathogenesis of autoimmune disease.

Studies with the mouse model of spontaneous murine autoimmune disease indicate that increased NO production corresponds with the onset of autoimmune disease and the manifestations of the disease can be reduced by administering a NO inhibitor (Weinberg et al., J. Exp. Med. 179:651-660 (1994)). Abnormalities described in tumor vasculature are attributed to increased NO production in the tumor. NO production is also associated with increased vascularization in nude mice resulting in rapid progression of tumor growth (Andrade et al., Br. J. Pharmacol. 107:1092-1095 (1992)). In addition, NO affects immune suppression in transplantation and graft rejection. Increased NO levels correlate to the degree of graft rejection (Tanaka et al., Transplantation 60:713-717 (1995); Devlin et al., Transplantation 58:592-595 (1994)). Use of the immunosuppressive drugs cyclosporin A or FK506 result in the inhibition of NO production in vivo (Langrehr et al., J. Clin. Invest. 90:679-683 (1992)). In addition, aminoguanidine administration selectively inhibits iNOS and was beneficial to survival (Devlin et al., supra, 1994), while non-specific NOS inhibitors were not beneficial (Drobyski et al., Blood 84:2363-2373 (1994)). Therefore, selective regulation of iNOS is important in treating many disease conditions as well as successful transplantation and graft procedures.

Excessive NO levels in tissues lead not only to injury of a tissue or organism, but also down regulate cNOS (Griscavage et al., Adv. Pharmacology 34:215-234 (1995)). Septic shock, for example, occurs when microbial products trigger systemic inflammatory responses. Endotoxin, and the resultant induction of inflammatory cytokines, causes a dramatic non-responsive fall in central blood pressure, eventual organ failure and death. iNOS is an important contributor to this hyporesponsive systemic hypotension. However,

administration of non-specific NOS inhibitors worsen outcome by inhibiting cNOS, which is necessary for cardiovascular regulation (Laszlo et al., Br. J. Pharmacol. 111 (4):1309-1315 (1994); Harbrecht et al., J. Leukocyte Biol. 52 (4):390-394 (1992)). Increased production of NO also contributes to tissue damage in a variety of inflammatory conditions such as immune injury to the kidney. Kidney proximal tubules produce arginine, the precursor of NO. The kidney is susceptible to high levels of NO production in the presence of high local arginine concentrations. cNOS inhibition by NO generated by iNOS also provokes renal dysfunction in rats treated with LPS (Schwartz et al., J. Clin. Invest. 100:439-448 (1997)). Therefore, it is advantageous to selectively modify iNOS activity without altering the constitutive NO levels required for normal homeostasis and protective capacity.

ADC coverts arginine to agmatine. The ADC activity is indicative of agmatine production in various tissues, such as in membrane-enriched fractions of the brain, liver, and kidney cortex and medulla (Lortie et al., supra, 1996). Constitutive ADC activity in mammalian systems is highest in the kidney (glomeruli and tubules) and the liver (Lortie et al., supra, 1996). Arginine is the only physiological nitrogen donor for the NOS catalyzed NO synthesis.

Proximal tubules are a major site of arginine synthesis in the kidney (Levillian et al., Am. J. Physiol. 259(28):F916-F923 (1990)). Therefore, a mouse kidney proximal tubule cell line, MCT (Olanrewaju et al., supra, 1992, which is incorporated herein by reference), was used as a model to determine if metabolites of the ADC pathway could modulate NO production. Arginine derivatives, as well as various polyamines, were tested for their ability to inhibit iNOS activity in response to

cytokine administration to the cells. Agmatine is structurally similar to the polyamine putrescine, being composed of two cationic regions separated by a four carbon chain backbone. It differs from the polyamines in having a guanidinium moiety as one cationic moiety.

Regulation of iNOS by ADC generated arginine metabolites in cells is disclosed herein (see Example V). These metabolites include the arginine metabolite agmatine and the agmatine metabolite agmatine-aldehyde. Cytokine-stimulated MCT cells produced NO end products as determined by the Greiss reaction (see below). When compared with the unstimulated control cells, the NO end products of the stimulated cells correspond to NO produced by iNOS. As disclosed herein, agmatine addition to the cells, inhibited NO end product accumulation (Example V). The addition of DAO resulted in the reduction of NO end products, and DAO with agmatine further reduced NO endproduct accumulation. In contrast, the addition of AldDH increased the amount of NO end products. These results indicate that a metabolite of agmatine, agmatine-aldehyde, was an active agent in repressing NO end products from iNOS. The polyamines spermine and spermidine also were potent inhibitors of iNOS (Example V). However, these polyamines were toxic to cells at dosages greater than 100 μM, and their aldehyde derivatives were even more toxic (Example V). In contrast, agmatine is not toxic to cells or animals, even at multiple dosages of 80 mg/kg.

Several cell lines were screened for the efficacy of agmatine derivatives in reducing cytokine-stimulated iNOS production. iNOS production was reduced in all cell lines tested by exogenous agmatine addition (Example V). Variation in the transport of exogenous agmatine into the cells is responsible for the difference in efficacy.

Bacterial LPS was administered to Wistar Fromter rats as an in vivo model of septic shock. Multiple agmatine doses as high as 80 mg/kg administered intraperitoneally were well tolerated by the animals. Blood pressure and GFR, an indicator of kidney function, were both normalized by administration of agmatine. Thus, the extreme hypotension encountered in septic shock was normalized by agmatine. Furthermore, the animals had no side effects in response to agmatine administration and were alert and active afterwards. Schwartz et al., (Am. J. Physiol. 272:F597-F601 (1997) which is incorporated herein by reference) determined that agmatine increases GFR via a cNOS-dependent mechanism that is independent of interaction with α-adrenergic receptors.

The present invention provides a method of specifically inhibiting iNOS without inhibiting cNOS by administering an arginine derivative such as agmatine or a derivative thereof to a mammal. The present invention further provides a pharmacological composition containing an arginine derivative in a physiologically acceptable carrier. Arginine derivatives preferred in the present invention include agmatine and its metabolite agmatine-aldehyde.

A pharmacological composition of the invention can include other components enhancing the effectiveness or stability of the active agent. For example, the DAO enzyme co-administered with agmatine increases intracellular agmatine-aldehyde concentration derived from the agmatine administered and enhances the inhibitory effect on NO end product accumulation (see Example V). In fact DAO alone, decreases NO end product accumulation due to its action of producing agmatine-aldehyde. Inhibitors of AldDH also serve to increase the cellular pool of agmatine-aldehyde by

preventing breakdown of this metabolite to the acid. Examples of such inhibitors include DEAB (4-(diethylamine)-benzoaldehyde) and 4-methylpyrazol (Sigma Chemical Company). Thus, a pharmacological composition of the invention advantageously can include components stabilizing the pool of agmatine-aldehyde, such as DAO, an agonist of DAO, or an inhibitor of AldDH.

The present invention further provides a method of treating a pathological condition by administering a composition comprising an arginine derivative such as agmatine or a derivative of agmatine. Such a condition can be any pathological condition resulting, at least in part, from an excess of NO generation due to the stimulation of iNOS. Such pathological conditions include, for example, septic shock, arthritis, glomerulonephritis, angiogenesis in tumors, transplantation and graft rejection, neurodegeneration, stroke, ischemic injury, chronic inflammation and diabetes.

A method of the invention is particularly useful for treating septic shock. Until the present invention, there has been no satisfactory method of treating toxic shock. As used herein, the term "septic shock" refers to a condition of acute circulatory failure in a subject secondary to infection or the presence of toxic microbial products, for example, bacterial LPS, which trigger systemic inflammatory responses in the subject. Septic shock can be associated, for example, with hypotension, coldness of the skin, tachycardia and anxiety. Septic shock also can be associated, for example, with the induction of inflammatory cytokines, which trigger a dramatic fall in blood pressure, organ failure and death. The physiological response in septic shock is due to high NO levels due to iNOS induction. Therefore, the present invention provides a method of

treating septic shock by administering a pharmaceutical composition comprising an arginine derivative such as agmatine or a derivative thereof. Preferred dosages for the treatment of septic shock range from 5 µg/kg body weight to 80 mg/kg body weight.

In summary, the present invention provides methods of reducing polyamine levels and inhibiting iNOS activity by administering agmatine or a derivative thereof, thereby inducing AZ. Administering agmatine both inhibits ODC and reduces polyamine uptake into cells. Further, the methods of the invention inhibit iNOS activity while maintaining or enhancing cNOS activity. Therefore, the method is useful therapeutically for controlling certain pathological conditions involving excessive cell proliferation or enlargement.

The following examples are intended to illustrate but not limit the invention.

EXAMPLE I

Regulation of ODC Activity by Agmatine

A. Introduction

Arginine is converted to agmatine by ADC. ADC activity is considered indicative of agmatine production in various tissues (Lortie et al., supra, 1996), which is incorporated herein by reference. ADC activity has been demonstrated in the membrane-enriched fraction of the brain, liver, and kidney cortex and medulla by a radiochemical assay (Lortie et al., supra, 1996).

Experiments demonstrating the effect of agmatine on ODC activity in cells were performed both on isolated proximal tubules and MCT cell lines. The

preparation of these cells is described below. MCT cells demonstrate high constitutive ODC levels (Olanrewaju et al., supra, 1992).

Compensatory renal hypertrophy in response to unilateral nephrectomy is associated with early induction of ODC activity and increased polyamine levels, as described, for example, in Humphries et al., Am. J. Physiol. 255(24):F270-F277 (1988), which is incorporated herein by reference. A single kidney (unilateral nephrectomy) is removed from the experimental animal, and the remaining kidney experiences compensatory renal hypertrophy (increase in the size and weight of the kidney) in response. In response to a unilateral nephrectomy isolated tubule cells (prepared as described below) from the remaining kidney demonstrate marked changes in the activities of ODC and ADC as shown in the experiments described below.

B. Procedures

Tissue and Cell Line Preparation

The kidney cortex was dissected and separated into glomerular and tubule preparations by sequential sieving (Tucker et al., J. Clin. Invest. 78:680-688 (1986)). The preparations were then homogenized in ice-cold ODC reaction buffer (10 mM Tris, pH 7.4, 2.5 mM DTT, 0.3 mM pyridoxal-5-phosphate, and 0.1 mM EDTA). MCT cells (Olanrewaju et al., supra, 1992) were plated on 10 cm culture dishes and allowed to grow to confluence in DMEM with 5% FCS. Media was changed to AIM V (FCS free) media for all experimental incubations. The cells were harvested by washing with 10 ml ice-cold PBS, placed in an appropriate volume of ODC reaction buffer, scraped, collected and homogenized. Kidney or MCT cell preparations were then centrifuged at 30,000 x g for 40

minutes at 4°C. The supernatant was collected and 250 µl used, in triplicate, in the ODC assay. The pellet was resuspended in a volume of ADC reaction buffer (10 mM Tris, pH 8.5, 2.5 mM DTT, 0.5 mM pefabloc (Boehringer-Mannheim, Indianapolis, IN), and 0.8 mM $MgSO_4$) equivalent to the volume of supernatant removed. The resuspension was used in triplicate, in the ADC assay.

Assays for ODC and ADC Activity

Each tissue preparation (250 µl) was added to large bare glass reaction tubes. Ex vivo tubular or glomerular preparations were incubated with agmatine (1 mM) for 1 hour at 37°C prior to the start of the enzyme reactions. MCT cells were incubated with experimental agents in DMEM plus 5% FCS for 16 hours unless otherwise noted, ODC and ADC reactions were started by the addition of 0.1 µCi ^{14}C-carboxyl labeled L-ornithine or L-arginine, respectively, to the tissue preparations. Tubes were capped with rubber stoppers fitted with metabolic wells (KONTES) containing 250 µl of trapping agent (Solvable, Dupont Corporation, Boston, MA). Incubations were for 1 hour at 37°C. Reactions were stopped by injection of 200 µl of 50% TCA and allowed to equilibrate for an additional hour prior to counting trapped ^{14}C-CO_2.

DNA Synthesis Assay

MCT cells were plated at 5000 cells/well in DMEM with 0.5% FCS and allowed to grow for 48 hours in the absence (control) or presence of agmatine (1 mM), or agmatine with putrescine (50 µM). Cells were labeled with ^{3}H-thymidine (1 µCi/well) for 18 hours prior to harvesting. Samples were counted in a scintillation counter.

C. Results

Tubular cells demonstrated marked inverse changes in the ODC and ADC activities in control versus nephrectomized tubule preparations (Figure 1). ODC activity increased in tubules 24 hours post-nephrectomy, while ADC activity was suppressed post-nephrectomy. Nephrectomy-induced ODC activity in tubules was blunted by the addition of agmatine ex vivo (Figure 2). Tubules were incubated for one hour with 1 mM agmatine or 1 mM putrescine.

The change in ODC activity of transformed MCT proximal tubule cells incubated for 16 hours in the presence of various concentrations of agmatine (1 μM to 1 mM) is shown (Figure 3A). Agmatine (1 mM) almost completely suppressed ODC activity. A comparison between the effect of various inhibitors on ODC activity, where the inhibitors were incubated for 16 hours without arginine (Lane 2), with 10 mM DFMO (Lane 3), 1 mM agmatine (Lane 4), or 1 mM putrescine (Lane 5) is shown (Figure 3B). A time course (30 minutes to 24 hours) for agmatine inhibition of ODC activity is shown (Figure 4). The effect of CHX or actinomycin-D on agmatine inhibition of ODC activity is presented (Figure 5A). MCT cells were incubated with or without 100 μg/ml CHX in the presence or absence of agmatine. Agmatine effectively inhibited ODC activity by about 74.5% in the absence of CHX. In the presence of CHX the level of inhibition is reduced to about 19.4% of the control. Therefore, agmatine inhibition requires new protein synthesis. Agmatine effectively inhibits ODC activity in the absence (by 88.7%) or presence (by 97.0%) of actinomycin-D, indicating new transcription was not required for inhibition of ODC by agmatine (Figure 5B).

Agmatine inhibits ODC activity in a number of additional cell lines. All cell lines were grown as described above in the presence or absence of 1 mM agmatine. At 48 hours the cells were harvested and assayed for ODC activity. The inhibition was compared to the control untreated cells. The various cell lines include MDCK canine kidney epithelial-like cell line (ATCC, Rockville, MD), ENDO endothelial cell line (Hothofer et al., Lab. Invest. 69(2):183-192 (1993)), MCT cells (Haverty et al., supra, 1988), JS-1 cancer transformed Schwann cell line, MC mouse kidney mesangial cell line, NKD-49-fibroblast cell line, LLCPK pig kidney cell line, Hep-G2 cancer transformed liver hepatocyte cell line, J774 and RAW309 monocyte/macrophage cell lines (ATCC, Rockville, MD). The ODC activity in all cell lines was inhibited to between 75% and 100% of control activity.

The inhibition of ^{3}H-agmatine uptake in MCT cells (Figure 6A) shows a competitive inhibition of ^{3}H-agmatine uptake (transport) by polyamines (putrescine, spermidine and spermine); and by a polyamine transport inhibitor paraquat. The results demonstrate that competition for agmatine transport was specific for polyamines. Conversely, agmatine uptake was not competitively inhibited by the arginine cationic (system y+) transporter agents L-NMMA or ornithine, lysine or arginine, or by agmatine's stable acid metabolite guanidinobutyric acid (GBA). These results indicate that agmatine was not universally transported by cationic transporters and that it utilized the same transport system as polyamines. Preincubation of MCT cells in 1 mM agmatine followed by ^{3}H-agmatine uptake over time demonstrated that agmatine down regulates its own transporter in a time-dependent fashion (Figure 6B).

The effect of agmatine (1 mM) or agmatine plus putrescine (1 mM plus 50 μM) on DNA synthesis of MCT cells is shown by ^{3}H-thymidine incorporation at 48 hours after additions (Figure 7). The experiment shows that agmatine is capable of reducing DNA synthesis in MCT cells. DNA synthesis in MCT cells was inhibited by day two in the presence of exogenous agmatine. Inhibition of DNA synthesis by agmatine can be reversed by the addition of putrescine. Agmatine inhibition of ODC activity leads to polyamine depletion and cessation of proliferation. The addition of putrescine (50 μM) bypassed the agmatine inhibitory effect and re-established DNA synthesis.

In summary, MCT are transformed proximal tubule cells and demonstrate high constitutive ODC levels. A lag period of 30 minutes prior to agmatine-dependent inhibition of ODC activity (Figure 4) correlates with observed polyamine induction of AZ (Hayashi et al., Biochem. J. 306:1-10 (1995)). Furthermore, experiments with CHX and actinomycin-D demonstrate that ODC inhibition is dependent upon new protein synthesis, but not new transcription (Figures 5A and 5B). Polyamine activation of AZ displays these characteristics due to translational frame-shifting (Matsufuji et al., supra, 1995) indicating that agmatine acts by inducing AZ.

Agmatine transport proceeds in a time and dose dependent fashion in MCT cells. Since AZ is capable of inhibiting polyamine transport, experiments were performed to determine whether agmatine regulates its own transport. Agmatine down regulates its transport in a temporal manner (Figure 6B). These results are consistent with agmatine induction of AZ.

DNA synthesis studies utilizing ^{3}H-thymidine incorporation demonstrated inhibition in proliferation of MCT cells exposed to agmatine for 48 hours, as compared

to control MCT cells (Figure 7). These results indicate that polyamines maintain cellular reserves (Davis, J. Cellular Biochem. 44:199-205 (1990); Davis et al., Microbiological Reviews 56(2):280-290 (1992)) that allow them to pass through several cell cycles before depletion limits further cell division (Davis et al., supra, 1992). Agmatine is capable of inhibiting ODC and depleting polyamine stores. The inhibitory effect of agmatine can be bypassed in the presence of putrescine (Figure 7). These results demonstrate the specificity of the inhibition, and that agmatine is not toxic at these doses.

Several cell types have been examined to determine the effect of agmatine. The effect of agmatine on ODC inhibition and DNA synthesis are similar in all types of cells examined.

EXAMPLE II

Effect of Agmatine on ODC and ADC Activity

The following results demonstrate that agmatine has an inverse effect on ODC and ADC activity.

Trapping of ^{14}C-CO_2, ^{14}C-ornithine or ^{14}C-arginine from a 30,000 x g supernatant or pellet, was utilized to assess ODC or ADC activity, respectively. Glomerular and tubular preparations were separated by sequential sieving, ODC and ADC assays were performed as described above. There were no changes in glomerular ODC activity 24 hours after nephrectomy. Nephrectomy reduced tubule ADC levels by greater than 60% with an induction of ODC activity by greater than 250% (Table 1). Tubules are the most prominent region in renal hypertrophy. Addition of exogenous agmatine to intact post-nephrectomy tubules reduced ODC activity to near control levels (Table 1).

The MCT cells described above express high constitutive ODC and low ADC levels. Addition of agmatine or putrescine (1 mM) to these cells inhibited ODC levels by greater than 95% and 80%, respectively (Table 2). Agmatine acts in a dose-dependent and time-dependent manner.

TABLE 1

Tubules*:	Cont.	NFX
ODC:	784	2,002
ADC:	2,318	869

TABLE 2

MCT*:	Cont.	+ Agmatine	+Putrescine
ODC:	26,725	1,131	5,356
ADC:	126	99	117

* ^{14}C-CO_2 release in cpm/hr/g tissue (Table 1) or per 2 x 10^6 cells (Table 2).

These results show that a non-feedback dependent pathway exists for ODC regulation by agmatine. High constitutive levels of ADC activity in kidney proximal tubules act to suppress ODC activity in spite of high local ornithine substrate concentrations. De-regulation and expression of ODC activity following nephrectomy result from down-regulation of ADC activity, increasing the polyamine levels contributing to the compensatory renal hypertrophy.

EXAMPLE III

Agmatine Inhibition of Polyamine Transport

The following experiments were performed to determine whether agmatine competitively inhibits polyamine uptake into cells. As demonstrated above (Example I), agmatine uptake was inhibited by polyamines and paraquat, however agmatine uptake was not inhibited by L-NMMA, ornithine, arginine or by GBA. To confirm that

agmatine utilizes the same transport system as polyamines, the following experiments were performed.

The uptake of ^{3}H-putrescine into MCT cells was measured in the presence of increasing concentrations of agmatine (10 μM to 1000 mM), putrescine, spermidine, paraquat, ornithine, lysine, and arginine (Figure 8). Agmatine (10 μM) reduced uptake to about 90% of the control, while a higher concentration (1 mM) completely inhibited ^{3}H-putrescine uptake. Polyamines also inhibited putrescine uptake, while the amino acids did not.

In another experiment agmatine was preincubated with MCT cell cultures for various times, washed out of the cultures, and the uptake of ^{3}H-putrescine measured (Figure 9). The times indicated on the graph are the length of exposure to agmatine prior to a 15 minute pulse of ^{3}H-putrescine. The uptake decreased rapidly over the first two hours (Figure 9), and remained suppressed for the time measured (24 hours). These results show that agmatine is capable of down-regulating polyamine transport, even at low concentrations. In addition, agmatine is capable of increasing the inhibition of ^{3}H-putrescine uptake in a time-dependent manner, comparable to the regulation of polyamine transport. These results, along with the results given in Example I above, demonstrate that agmatine can not only regulate the biosynthesis of polyamines, but can regulate its transport.

The effect of agmatine on ^{3}H-putrescine transport in the presence or absence of CHX and actinomycin-D was examined (Figure 10).

^{3}H-putrescine uptake was measured in the presence or absence of agmatine, with either actinomycin-D (5 µg/ml) or CHX (100 µg/ml). The putrescine uptake is reduced by CHX plus agmatine, but uptake in the presence of actinomycin-D and agmatine was similar to agmatine alone (Figure 10). These results confirm earlier studies indicating that protein synthesis is necessary to block polyamine uptake. In the presence of actinomycin-D, agmatine inhibition is about 110 percent of control levels of inhibition in both transport and ODC inhibition. However, in the presence of CHX, both transport and ODC activity are about 25 percent of the control level of inhibition. Figure 11 summarizes transport (TSP) and ODC activity.

EXAMPLE IV

Agmatine Acts by Inducing AZ

The experiments described in Examples I to III above demonstrate that agmatine is capable of inhibiting both ODC activity and polyamine uptake into cells. The inhibition has been influenced by translation but not transcription. The following experiments demonstrate that agmatine acts by inducing the AZ.

Agmatine decreased ODC activity in a dose-dependent manner in MCT cells (Figure 12). Cells were grown to approximately 70% confluence in 10 cm dishes and medium changed approximately 24 hours prior to the addition of increasing concentrations of agmatine or the DAO inhibitor aminoguanidine. Twenty-four hours later, cells were harvested, extracts of the cells prepared by three cycles of freezing and thawing, the cell

extract suspended in 0.3 ml of 0.155 M KCl containing 1 mM DTT, and the extract was centrifuged at 3000 x g for 20 minutes. The supernatant was assayed for ODC inhibitory activity by adding 50 µl of the supernatant to the ODC reaction mixture, described in Example I above, to a final volume of 0.140 ml.

Agmatine induced the ODC inhibitory activity AZ (Figure 13). The activity was precipitated with anti-AZ antibody and neutralized with an AZ inhibitor, indicating that the inhibitor is AZ. The same procedure was used as described in Figure 12 except that the MCT extract was treated with 10 mM agmatine. In addition, part of the extract was treated with control IgG or anti-AZ antibody bound to an immunoadsorbent (Kanamota et al., J. Biol. Chem. 268(13):9393-9399 (1993), which is incorporated herein by reference). Twenty microliters of each extract was added to the ODC assay mixture with or without the AZ inhibitor (Ain) purified from rats (Fujita et al., Biochem. J. 204:647-652; Kitani et al., Biochem. Biophys. Acta 991:44-49 (1989) each of which is incorporated herein by reference) and assayed for ODC inhibitory activity.

EXAMPLE V

Inhibition of Cytokine-Induced NO End Product Generation in MCT Cells

All reagents used in the following experiments were obtained from Sigma Chemical Company unless otherwise noted.

Agmatine and various polyamines were tested for their ability to inhibit iNOS activity in response to cytokine administration in MCT cells (Figure 14). Tumor necrosis factor-α (TNF-α, 10 ng/ml) and interferon-γ (IFN-γ, 200 U/ml) (Boehringer Mannheim Biochemicals, Indianapolis, IN) stimulated iNOS in MCT cells (Olanrewaju et al., supra, 1992), which is incorporated herein by reference and MC cells (Wolf et al., Am. J. Path. 140:95-107 (1992)) which is incorporated herein by reference). These transformed cells typically exhibit low ADC activity. Cells were grown for 3 to 4 days until nearly confluent in 24 well plates. TNF-α, IFN-γ and agmatine were added in one ml DMEM plus 5% FCS. At 48 hours, the supernatant was collected and used as a sample. Samples were reduced by E. coli nitrate reductase, and the NO end products quantified by the Greiss reaction. The cells were washed and the protein content determined using a standard Lowry procedure. The Greiss reaction measures only NO_2^- (Granger et al., J. Immunol. 146:1294-1302 (1991); Southan et al., Biochem. Biophys. Res. Commun. 203:1638-1644 (1944), each is incorporated herein by reference). Nitrate is reduced to nitrite with bacterial nitrate reductase, and then nitrite is measured using the Greiss reagent (1% sulfanilamine and 0.1% naphthylethylenediamide in 5% phosphoric acid, as described in Report of the American Institute of Nutrition Ad hoc Committee on Standards for Nutritional Studies J. Nutr. 107:1340 (1977); Green et al., Anal. Biochem. 126:131 (1982), both of which are incorporated herein by reference). The optical density of the reaction mixture at 550 nm is measured. Northern blot analysis (Sambrook et al., Molecular Cloning: A Laboratory Manual, 2nd ed. Cold Spring Harbor

Laboratory Press (1989) which is incorporated herein by reference) was utilized to determine RNA levels of iNOS messenger RNA.

The addition of agmatine to MCT cells inhibited NO levels (Figure 14) as did spermidine and spermine. Putrescine, GBA, and ornithine had little effect on the NO levels in MCT cells. The MC cells were less responsive to inhibitors, a difference attributed to lower agmatine transport than in MCT cells. Non-activated MCT control cells produced undetectable levels of NO end products. Therefore, the cytokine-stimulated values shown represent the contribution of induced NOS end products (Figure 14).

Agmatine inhibition of cytokine-induced NO end product generation in MCT cells was dose-dependent (Figure 14). Spermidine inhibits iNOS in macrophages (Granger et al., supra, 1991), however, spermidine and spermine are cytotoxic at high concentrations. GBA and ornithine failed to inhibit NO generation in MCT cells. Putrescine demonstrated marginal inhibitory effects.

MCT cells were incubated with cytokines (as described above) for 24 hours followed by addition of exogenous agmatine (1 mM) at the times indicated (Figure 15). Agmatine addition prior to, during, or after cytokine addition resulted in a decrease in NO end product accumulation. These results show that the maximum inhibition is obtained when agmatine is administered two hours prior to the addition of cytokines. These results (Figures 14 and 15) demonstrate that agmatine inhibits iNOS activity in a dose-dependent and time-dependent manner.

Northern blot analysis was performed on the above samples. The mRNA for iNOS was not significantly inhibited throughout the time course relative to the control. These results indicate that the inhibition by agmatine or an agmatine derivative was not transcriptionally mediated, suggesting either post-translational modification or substrate competition as a mechanism of iNOS inhibition.

Addition of increasing concentrations of highly purified DAO (Novotny et al., J. Biol. Chem. 269(13):9921-9925 (1994)) to MCT cells at the time of cytokine stimulation decreased NO end product accumulation (Figure 16A). Increasing concentrations of DAO, added to MCT cell cultures with 1 mM agmatine, increased the inhibition of NO end product accumulation 48 hours after the addition (Figure 16B). These results indicate that the agmatine metabolite guanidinobutyraldehyde, is the causal agent inhibiting NO end product accumulation.

Pentamidine (Sigma Chemical Company) is an effective inhibitor of DAO, but has no inhibitory effect on NO end product accumulation. Agmatine and increasing concentrations of pentamidine were administered to MCT cell cultures (Figure 17). Agmatine, pentamidine and cytokines were added and the cells incubated for 48 hours, samples of media were taken, and the samples analyzed for total nitrite by the Greiss reaction. Protein content of the cells was determined by the Lowry reaction. Pentamidine repressed the inhibitory effects of agmatine on iNOS (Figure 17). Pentamidine blocks the conversion of agmatine to agmatine-aldehyde by DAO. Increasing

concentrations of agmatine can overcome the repression of pentamidine (Figure 18).

Increasing concentrations of AldDH added to MCT cell cultures without agmatine increased the amount of cytokine-induced NO end products (Figure 19A). The AldDH co-factor, NAD (1 mM), increased NO end product accumulation over the control (Figure 19A). Agmatine (1 mM) with increasing concentrations of AldDH decreased the inhibition of NO end product accumulation (Figure 19B). These results show that the level of agmatine-aldehyde in the cell can be modulated by adding an enzyme or cofactor of the enzyme that metabolizes agmatine-aldehyde, thereby altering the amount of iNOS end product generated. Increasing the agmatine concentration in the assay (0-2.5 mM) can overcome the effect of the added AldDH (Figure 20).

Various cell lines were stimulated to induce iNOS activity for 48 hours and nitrite end products measured by the Greiss reaction described above. Cell lines include NRK-49 fibroblast cells, J774 monocyte cell cultures (ATCC, Rockville, MD), mesangial cell lines, endothelial cell lines (Hothofer et al., supra, 1993) and MCT cells (Haverty, et al., supra, 1988). Cells were grown as described above, and increasing concentrations of agmatine were added to the cell lines. All of the cell lines showed a decrease in NO end product accumulation compared to the control with increasing concentrations of agmatine (Figure 21). The response to agmatine, however, varies substantially between cell types, due to variations in agmatine uptake in the different cells.

EXAMPLE VI

Treatment of Endotoxic Shock in Animals

Eight Wistar Promter rats were divided into two groups. Four rats were injected intraperitoneally with 0.5 mg/kg bacterial LPS (Sigma Chemical Company), and four additional rats received 80 mg/kg agmatine along with the LPS as an intraperitoneal injection. A second agmatine dose of 80 mg/kg was administered after six hours. Clearance studies were performed to determine GFR (Blantz et al., Methods in Pharmacology, vol. 4B, Renal Pharmacology, M. Martinez-Madonado, Plenum Press, NY (1978), and the blood pressure of the animals was monitored after eight hours. Blood pressure (Figure 22A) and GFR (Figure 22B) were restored in animals administered both LPS and agmatine. The animals did not suffer any side effects from the agmatine, and were alert and active after administration of high dosages of the compound.

Although the invention has been described with reference to the examples above, it should be understood that various modifications can be made without departing from the spirit of the invention. Accordingly, the invention is limited only by the following claims.

We claim:

1. A method of reducing the intracellular concentration of polyamines in a cell, comprising administering a composition comprising an arginine derivative to the cell.

2. The method of claim 1, wherein said arginine derivative is agmatine.

3. The method of claim 1, wherein the composition further comprises a diamine oxidase inhibitor.

4. The method of claim 3, wherein said diamine oxidase inhibitor is aminoguanidine or pentamidine.

5. A method of inhibiting ornithine decarboxylase activity in a cell, comprising administering a composition comprising an arginine derivative to the cell.

6. The method of claim 5, wherein said arginine derivative is agmatine.

7. A pharmacological composition that reduces polyamine levels intracellularly, comprising an arginine derivative in a physiologically acceptable buffer.

8. The pharmacological composition of claim 7, wherein said arginine derivative is agmatine.

9. The pharmacological composition of claim 7, further comprising a diamine oxidase inhibitor.

10. A method of treating a pathological condition involving abnormal cell proliferation or enlargement, comprising contacting the cells with a composition comprising an arginine derivative.

11. The method of claim 10, wherein said arginine derivative is agmatine.

12. The method of claim 10, wherein the pathological condition is tumor growth.

13. The method of claim 10, wherein the pathological condition is angiogenesis.

14. The method of claim 10, wherein the condition is selected from the group consisting of cardiac hypertrophy, renal disease progression and renal disease due to diabetes.

15. The method of claim 10, wherein the pathological condition is clonal cell expansion in an immune response.

16. A method of inhibiting inducible nitric oxide synthase in a mammal while maintaining constitutive nitric oxide synthase, comprising administering a composition comprising an arginine derivative to the mammal.

17. The method of claim 16, wherein said arginine derivative is agmatine.

18. The method of claim 16, wherein said arginine derivative is agmatine-aldehyde.

19. The method of claim 16, wherein said composition further comprises diamine oxidase or an agonist thereof.

20. The method of claim 16, wherein said composition further comprises an inhibitor of aldehyde dehydrogenase.

21. A pharmacological composition that selectively inhibits inducible nitric oxide synthase in a mammal without inhibiting constitutive nitric oxide synthase, comprising an arginine derivative in a physiologically acceptable carrier.

22. The pharmacological composition of claim 21, wherein said arginine derivative is agmatine.

23. The pharmacological composition of claim 21, wherein said arginine derivative is agmatine-aldehyde.

24. A method of treating septic shock in a mammal, comprising administering a composition comprising an arginine derivative to a mammal.

25. The method of claim 24, wherein said arginine derivative is agmatine.

26. The method of claim 24, wherein said arginine derivative is agmatine-aldehyde.

27. The method of claim 24, wherein said arginine derivative is administered in a dosage of between 5 µg/kg body weight to 80 mg/kg body weight.

28. A pharmacological composition that restores blood pressure and glomerular filtration rate during septic shock, comprising an arginine derivative and a physiologically acceptable carrier.

29. The pharmacological composition of claim 28, wherein said arginine derivative is agmatine.

30. The pharmacological composition of claim 28, wherein said arginine derivative is agmatine-aldehyde.

31. The pharmacological composition of claim 28, wherein said composition further comprises diamine oxidase.

32. The pharmacological composition of claim 28, wherein said composition further comprises an inhibitor of aldehyde dehydrogenase.

33. A method of treating a condition in a mammal resulting from excessive nitric oxide production due to inducible nitric oxide synthase activity, comprising administering a composition comprising an arginine derivative to the mammal.

34. The method of claim 33, wherein said condition is selected from the group consisting of arthritis, glomerulonephritis, angiogenesis in tumors, transplantation and tissue graft rejection, neurodegeneration, stroke, ischemic injury, chronic inflammation and diabetes.

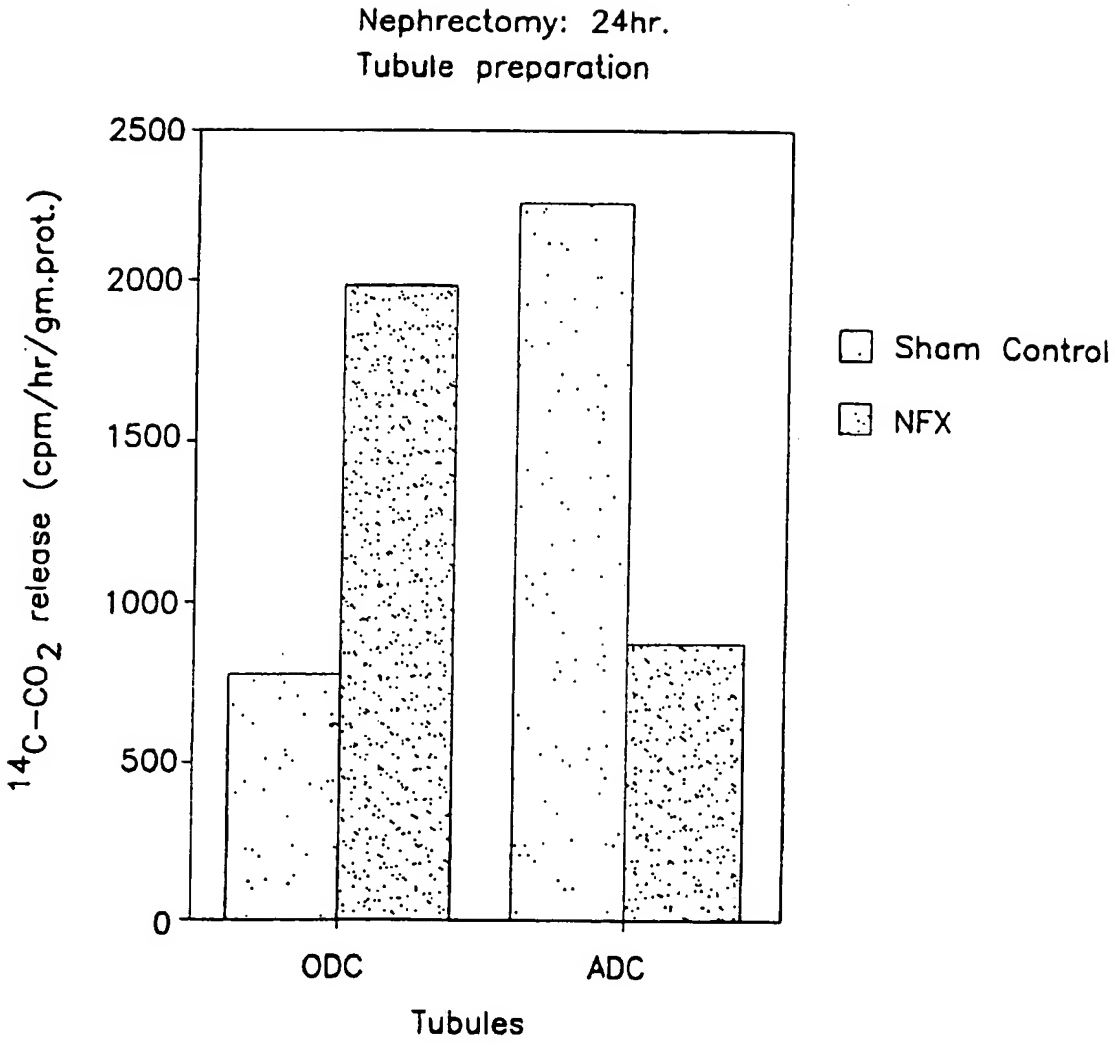

Nephrectomy: 24hr.
Tubule preparation
2500
2000
1500
1000
500
0
$^{14}C-CO_2$ release (cpm/hr/gm.prot.)
ODC
ADC
Tubules
Sham Control
NFX

FIG. 1

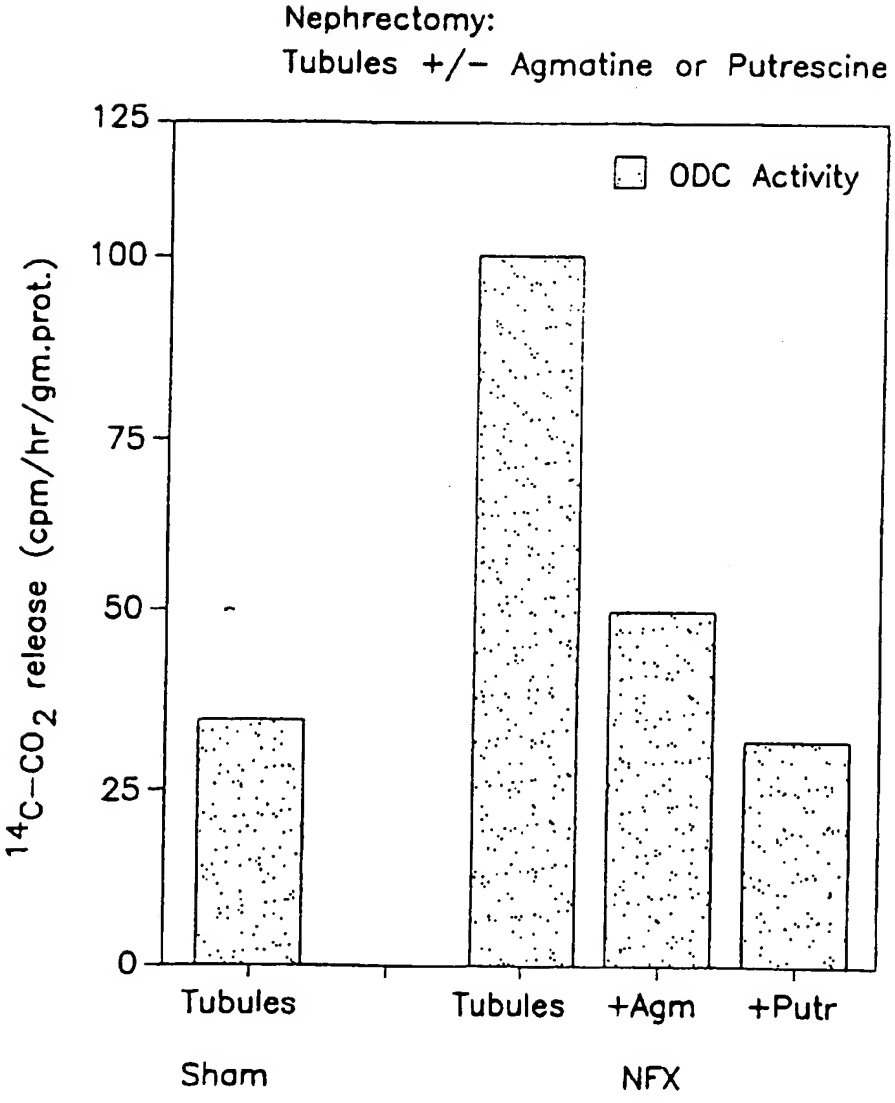

Nephrectomy:
Tubules +/− Agmatine or Putrescine
ODC Activity
$^{14}C-CO_2$ release (cpm/hr/gm.prot.)
125
100
75
50
25
0
Tubules
Tubules
+Agm
+Putr
Sham
NFX

FIG. 2

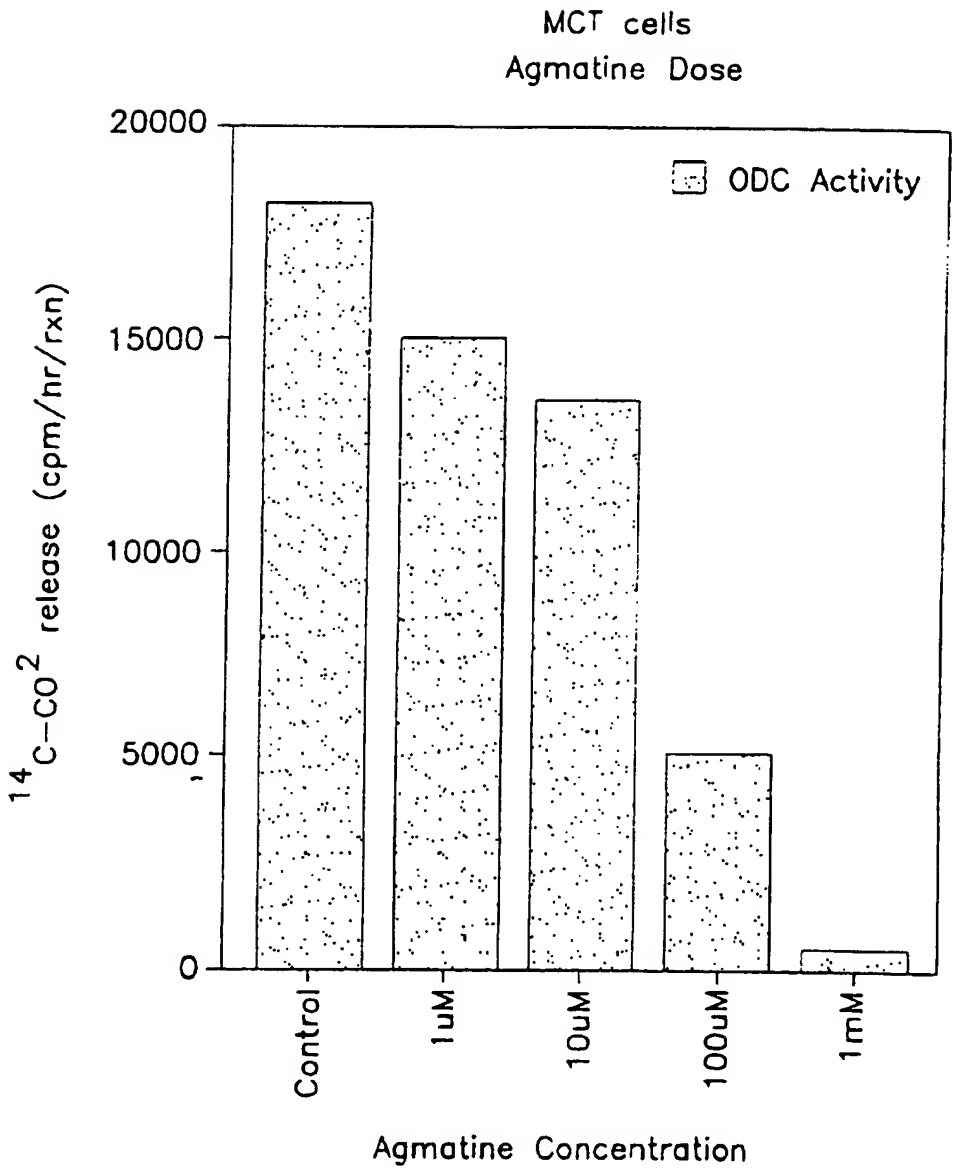

MCT cells
Agmatine Dose
ODC Activity
20000
15000
10000
5000
0
$^{14}C-CO^2$ release (cpm/hr/rxn)
Control
1uM
10uM
100uM
1mM
Agmatine Concentration

FIG. 3A

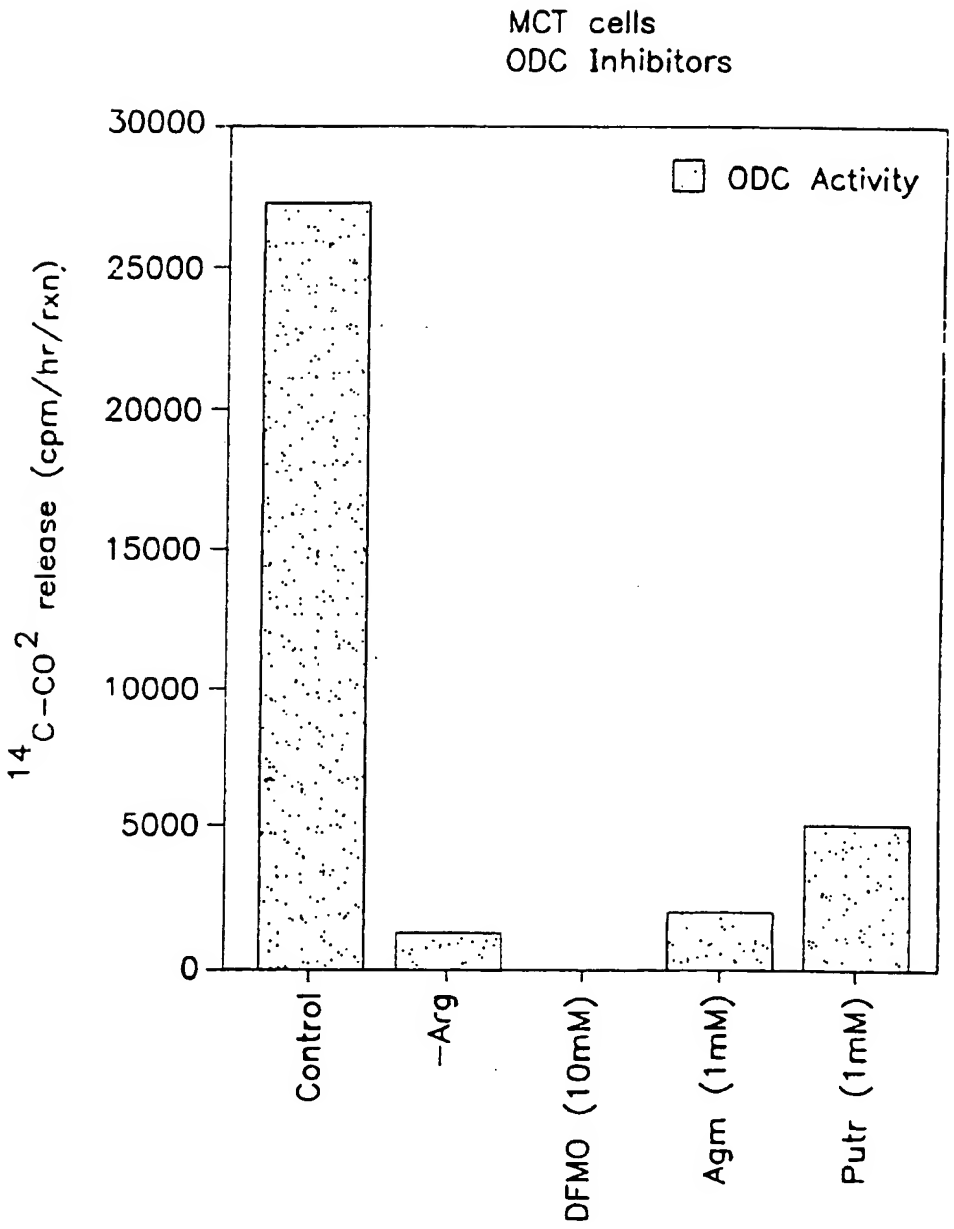

MCT cells
ODC Inhibitors
ODC Activity
$^{14}C-CO^2$ release (cpm/hr/rxn)
30000
25000
20000
15000
10000
5000
0
Control
-Arg
DFMO (10mM)
Agm (1mM)
Putr (1mM)

FIG. 3B

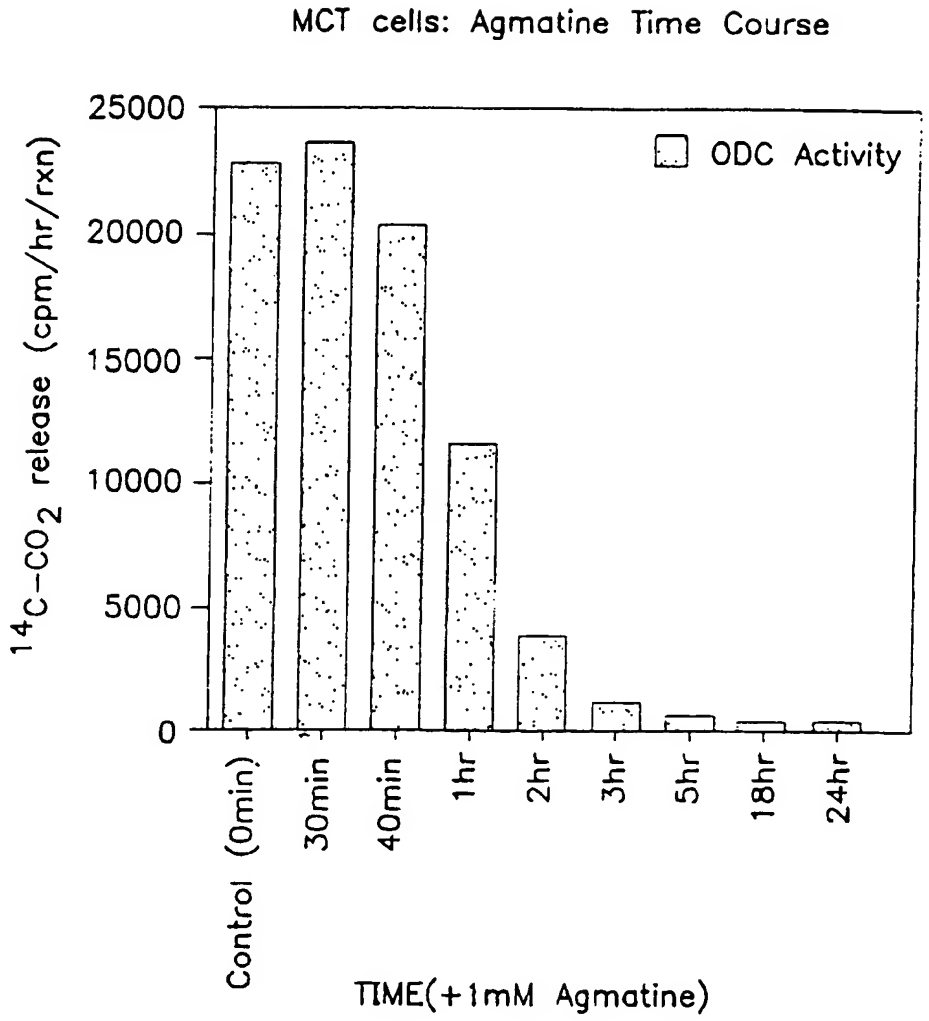

MCT cells: Agmatine Time Course
25000
20000
15000
10000
5000
0
$^{14}C-CO_2$ release (cpm/hr/rxn)
ODC Activity
Control (0min)
30min
40min
1hr
2hr
3hr
5hr
18hr
24hr
TIME(+1mM Agmatine)

FIG. 4

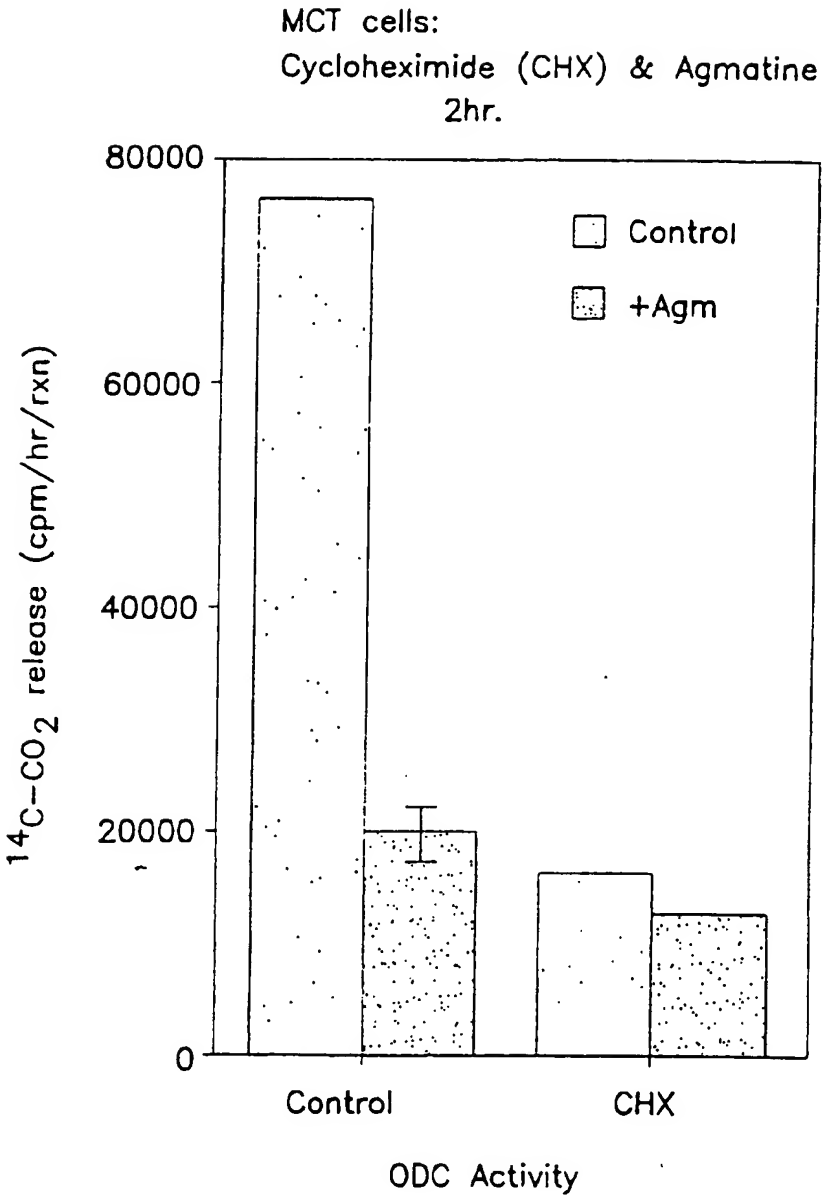

MCT cells:
Cycloheximide (CHX) & Agmatine
2hr.
80000
60000
40000
20000
0
$^{14}C-CO_2$ release (cpm/hr/rxn)
Control
+Agm
Control
CHX
ODC Activity

FIG. 5A

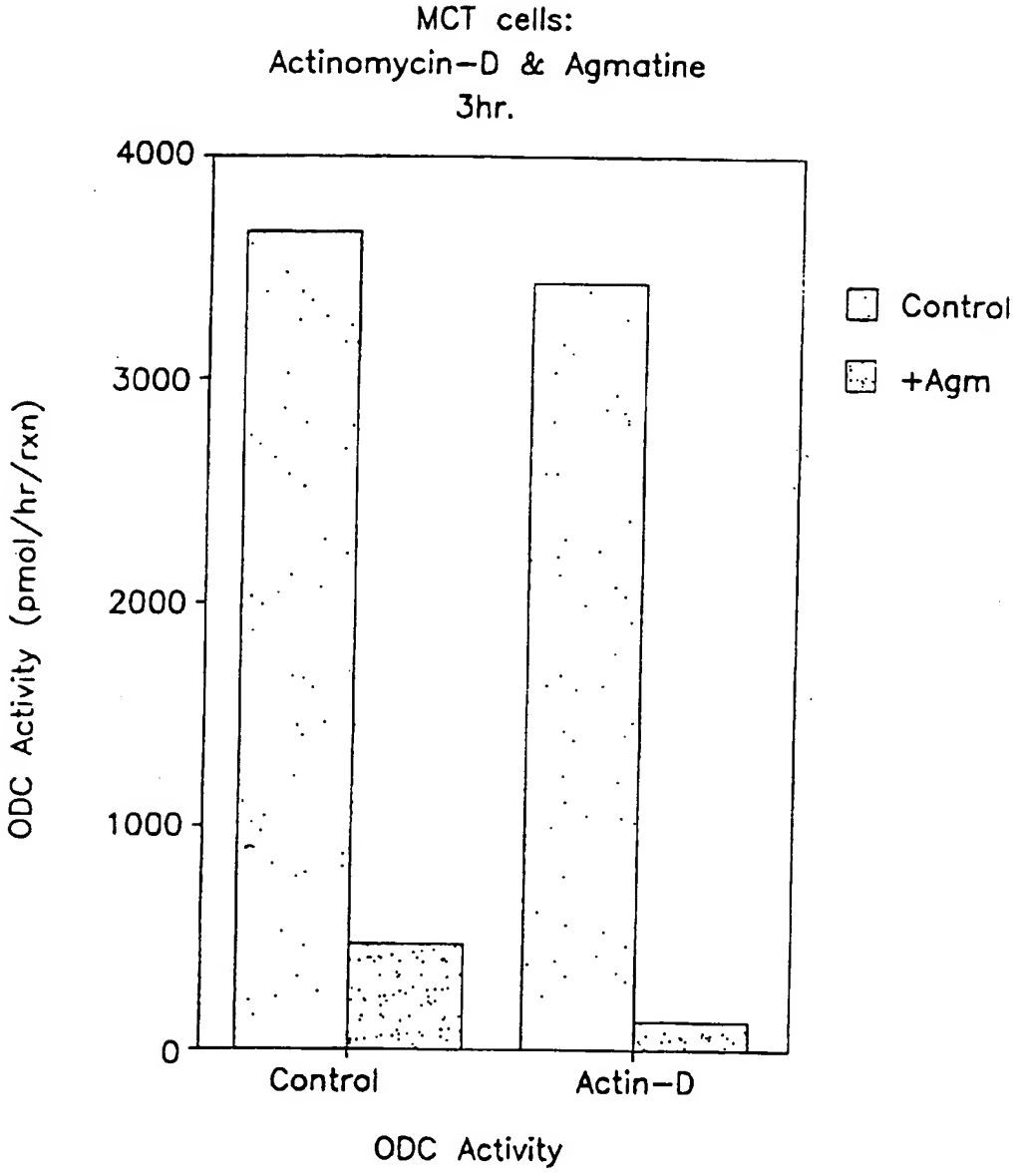

MCT cells:
Actinomycin–D & Agmatine
3hr.
4000
3000
2000
1000
0
ODC Activity (pmol/hr/rxn)
Control
+Agm
Control
Actin–D
ODC Activity

FIG. 5B

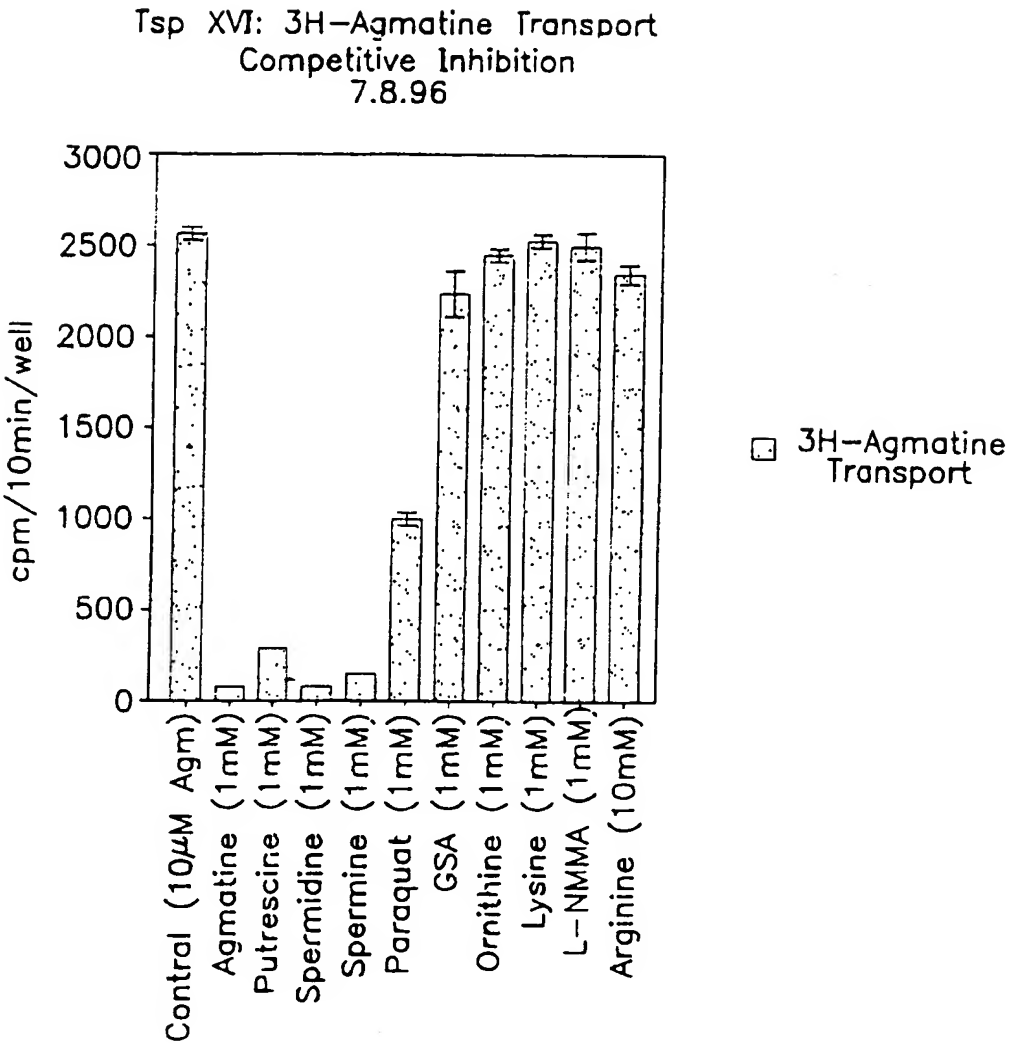

Tsp XVI: 3H-Agmatine Transport
Competitive Inhibition
7.8.96
cpm/10min/well
3000
2500
2000
1500
1000
500
0
Contral (10μM Agm)
Agmatine (1mM)
Putrescine (1mM)
Spermidine (1mM)
Spermine (1mM)
Paraquat (1mM)
GSA (1mM)
Ornithine (1mM)
Lysine (1mM)
L-NMMA (1mM)
Arginine (10mM)
3H-Agmatine Transport

FIG. 6A

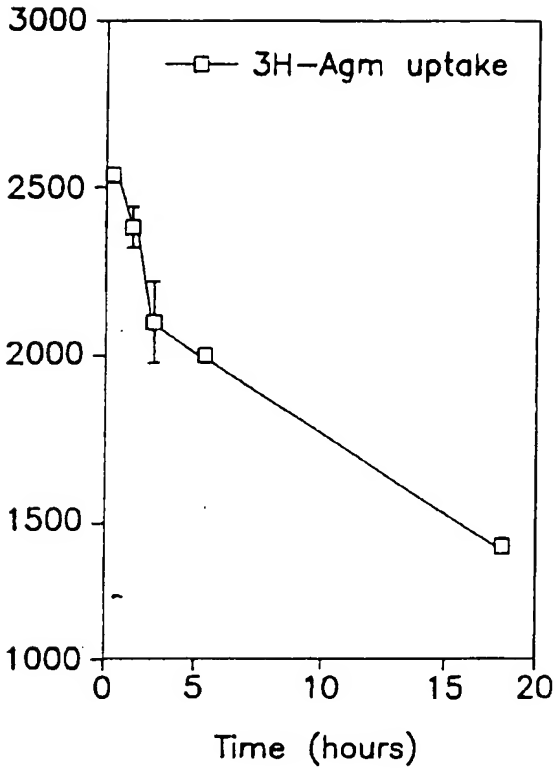


FIG. 6B

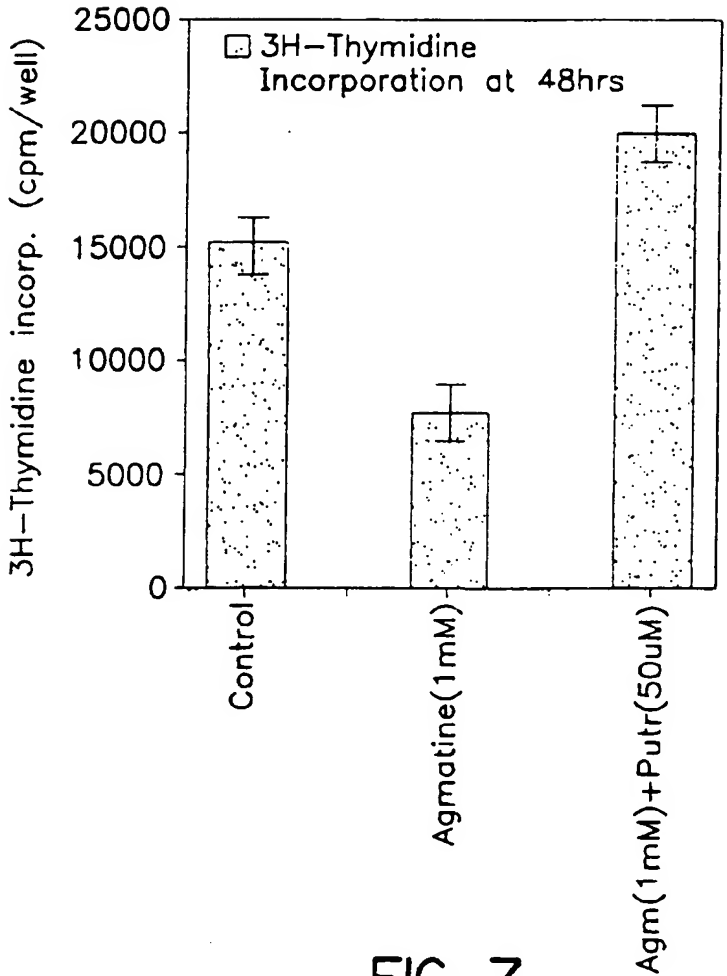

25000
20000
15000
10000
5000
0
3H-Thymidine incorp. (cpm/well)
3H-Thymidine Incorporation at 48hrs
Control
Agmatine(1mM)
Agm(1mM)+Putr(50uM)

FIG. 7

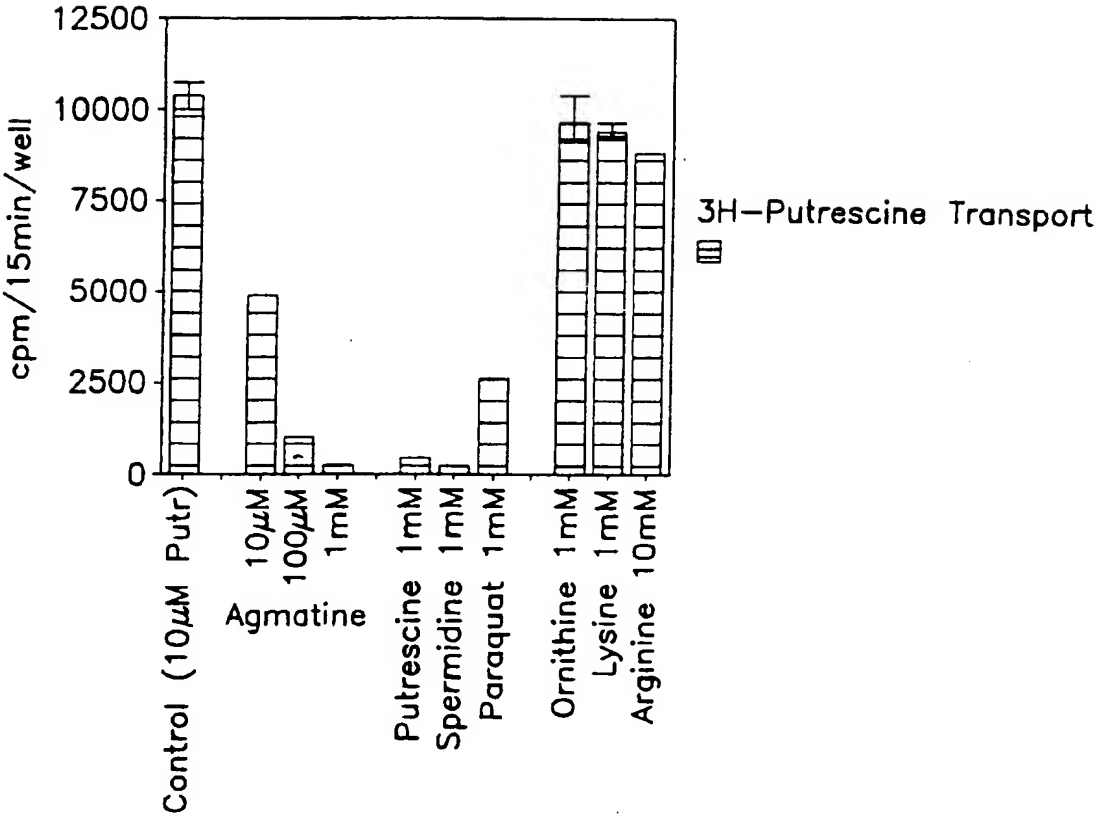

12500
10000
7500
5000
2500
0
cpm/15min/well
3H–Putrescine Transport
Control (10μM Putr)
10μM
100μM
1mM
Agmatine
Putrescine 1mM
Spermidine 1mM
Paraquat 1mM
Ornithine 1mM
Lysine 1mM
Arginine 10mM

FIG. 8

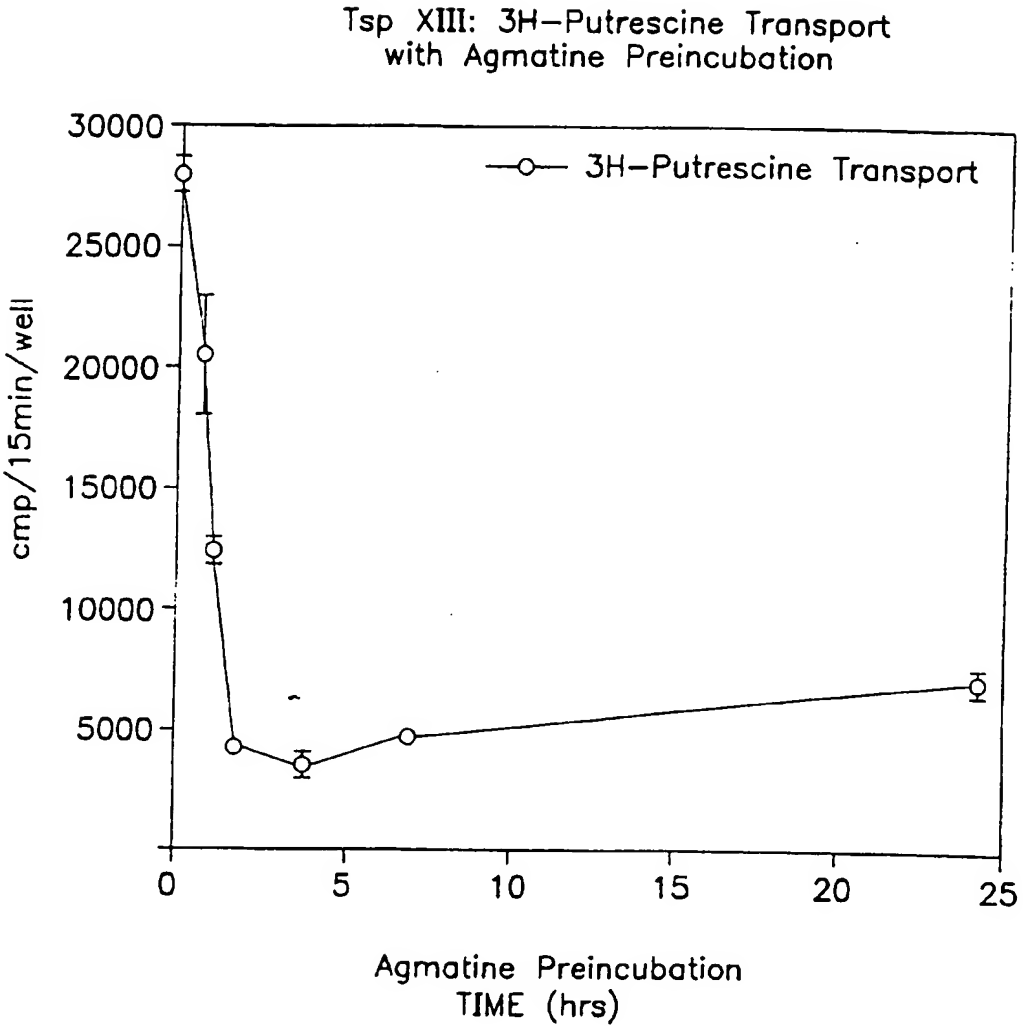
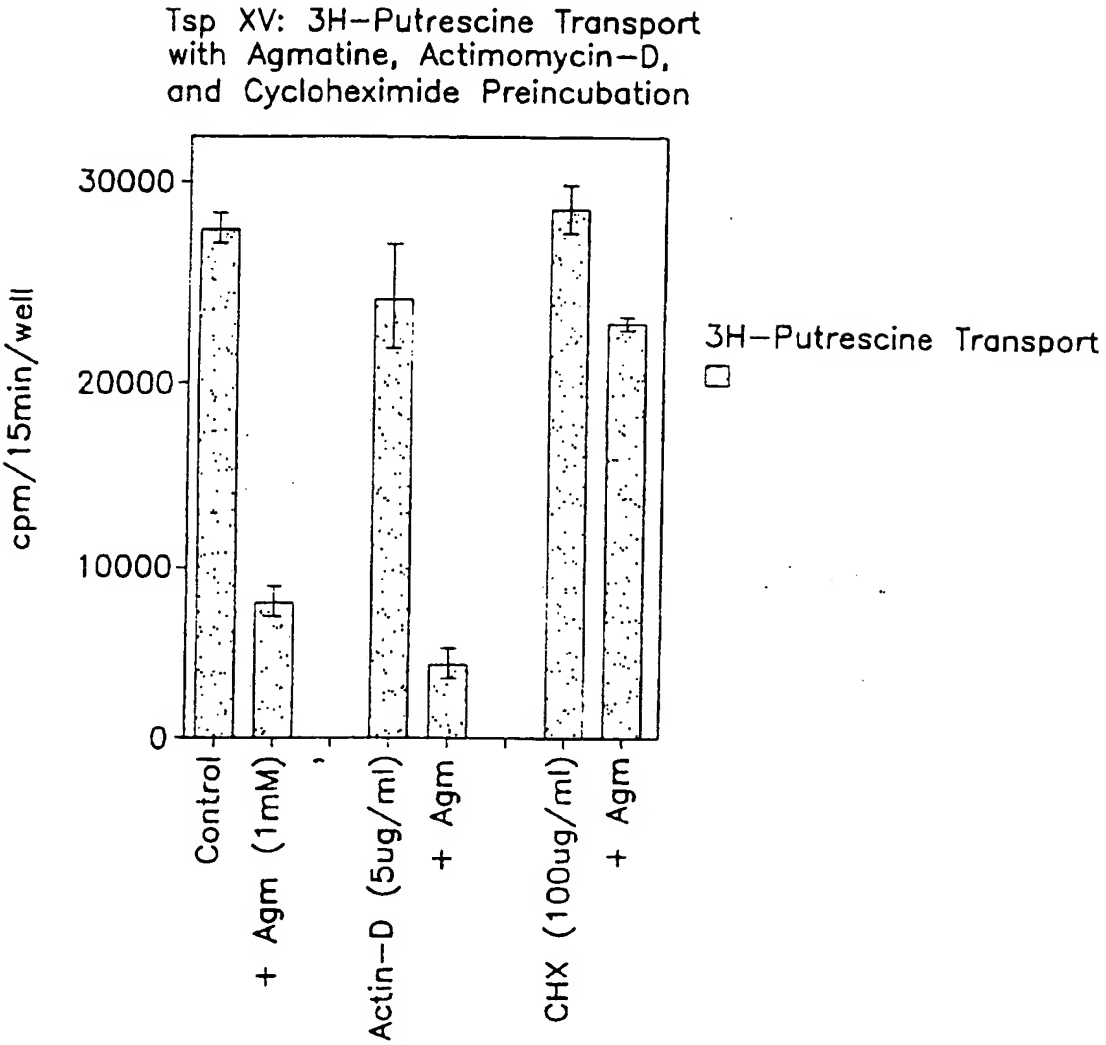

Tsp XIII: 3H–Putrescine Transport
with Agmatine Preincubation
3H–Putrescine Transport
cmp/15min/well
30000
25000
20000
15000
10000
5000
0
5
10
15
20
25
Agmatine Preincubation
TIME (hrs)

FIG. 9

FIG. 10


Tsp XV: 3H-Putrescine Transport
with Agmatine, Actimomycin-D,
and Cycloheximide Preincubation
cpm/15min/well
30000
20000
10000
0
Control
+ Agm (1mM)
Actin-D (5ug/ml)
+ Agm
CHX (100ug/ml)
+ Agm
3H-Putrescine Transport

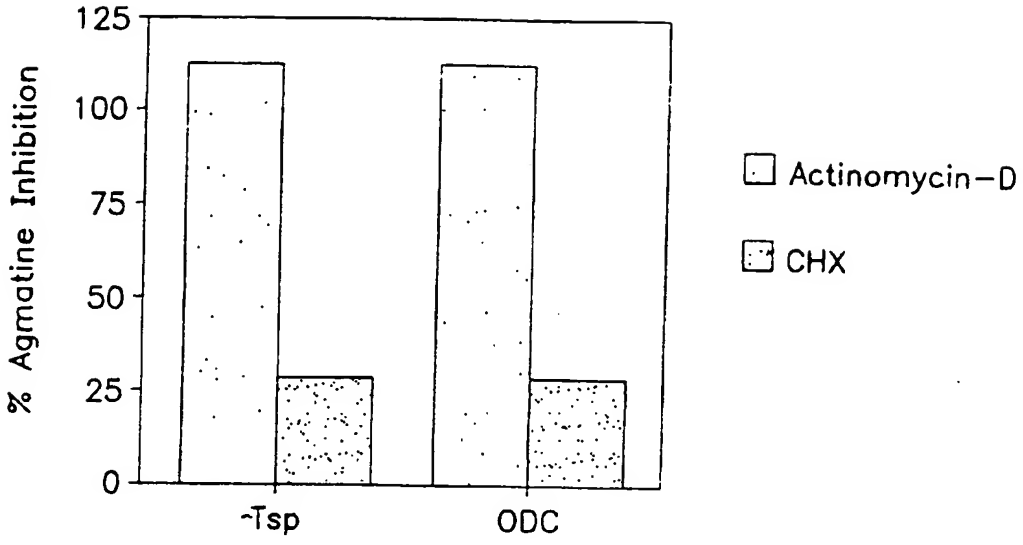

125
100
75
50
25
0
% Agmatine Inhibition
-Tsp
ODC
Actinomycin-D
CHX

FIG. 11

Effect of extract of MCT cells treated with various concentrations of agmatine on ODC activity

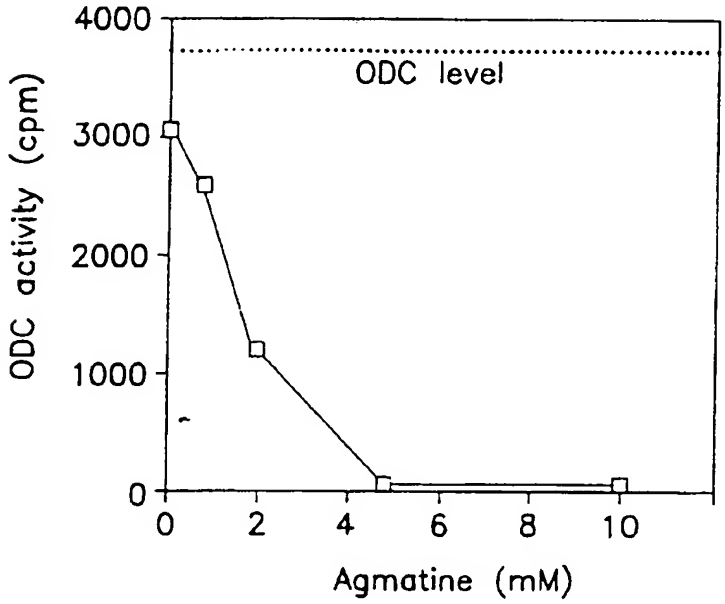


FIG. 12

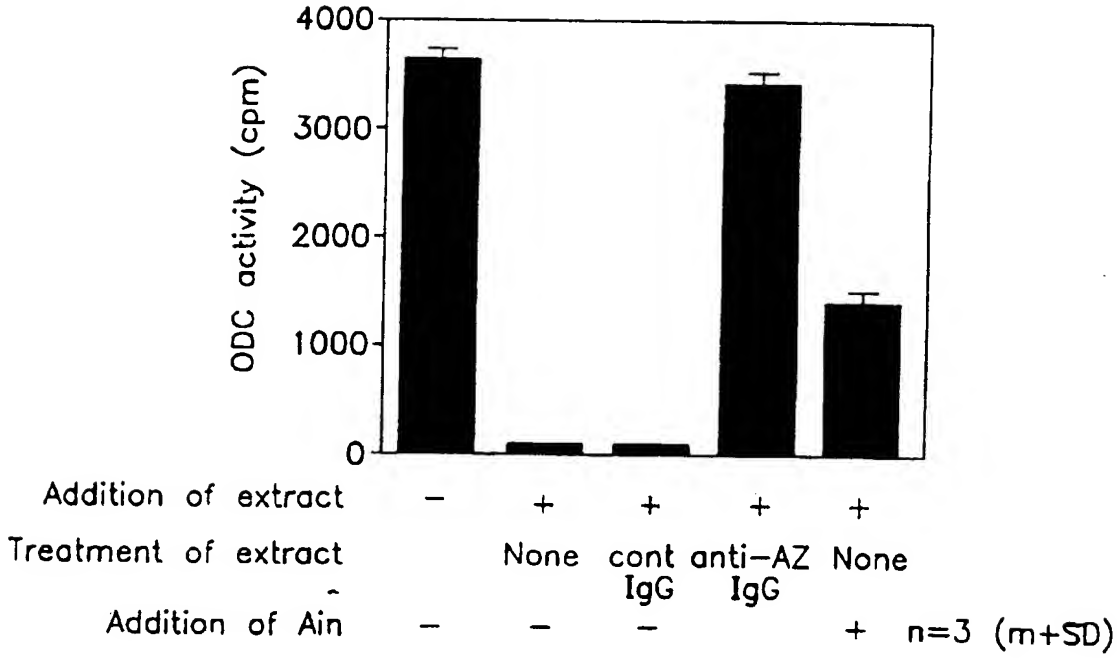

4000
3000
2000
1000
0
ODC activity (cpm)
Addition of extract – + + + +
Treatment of extract None cont IgG anti-AZ IgG None
Addition of Ain – – – + n=3 (m+SD)

FIG. 13

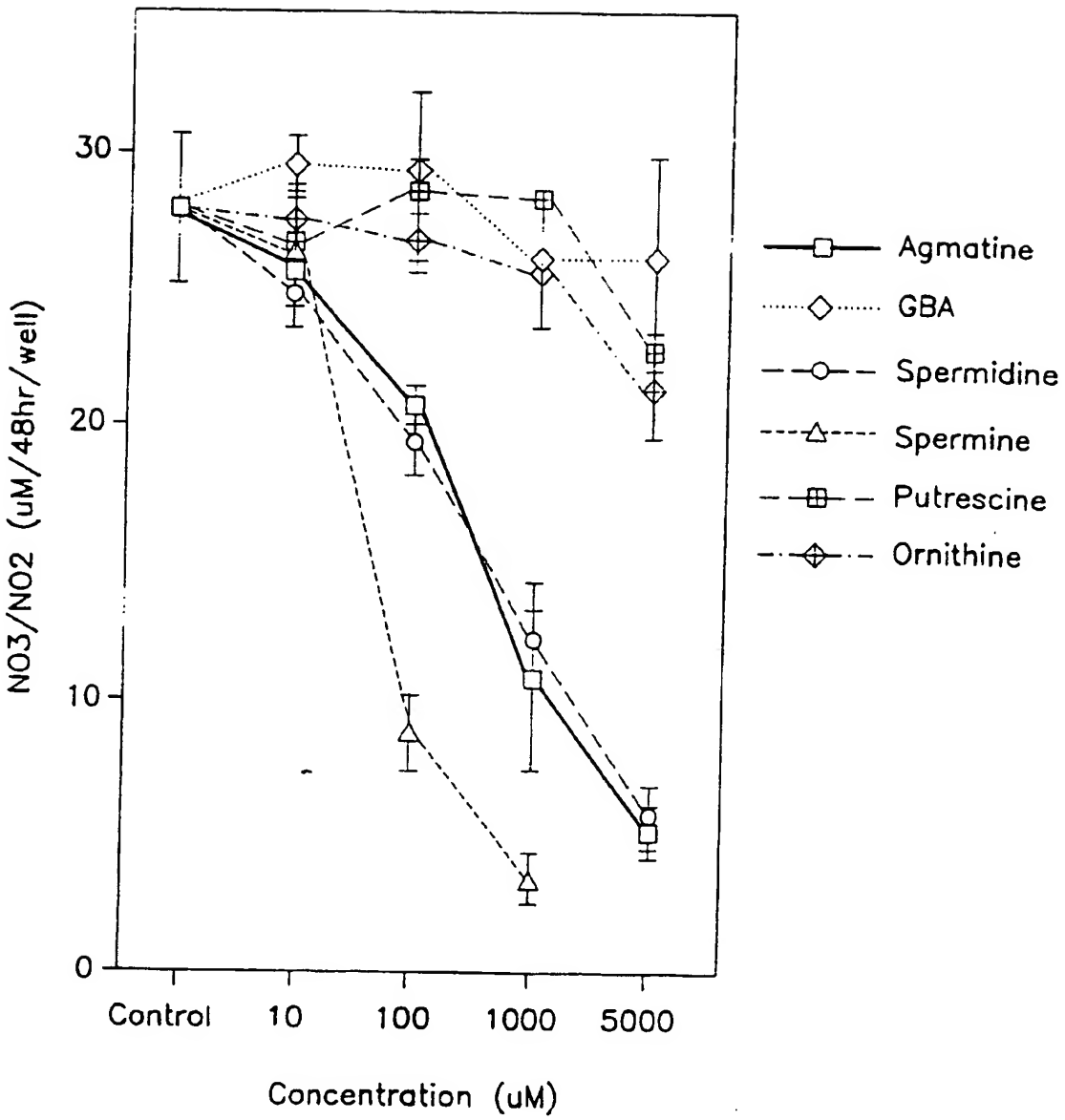

30
20
10
0
NO3/NO2 (uM/48hr/well)
Control
10
100
1000
5000
Concentration (uM)
Agmatine
GBA
Spermidine
Spermine
Putrescine
Ornithine

FIG. 14

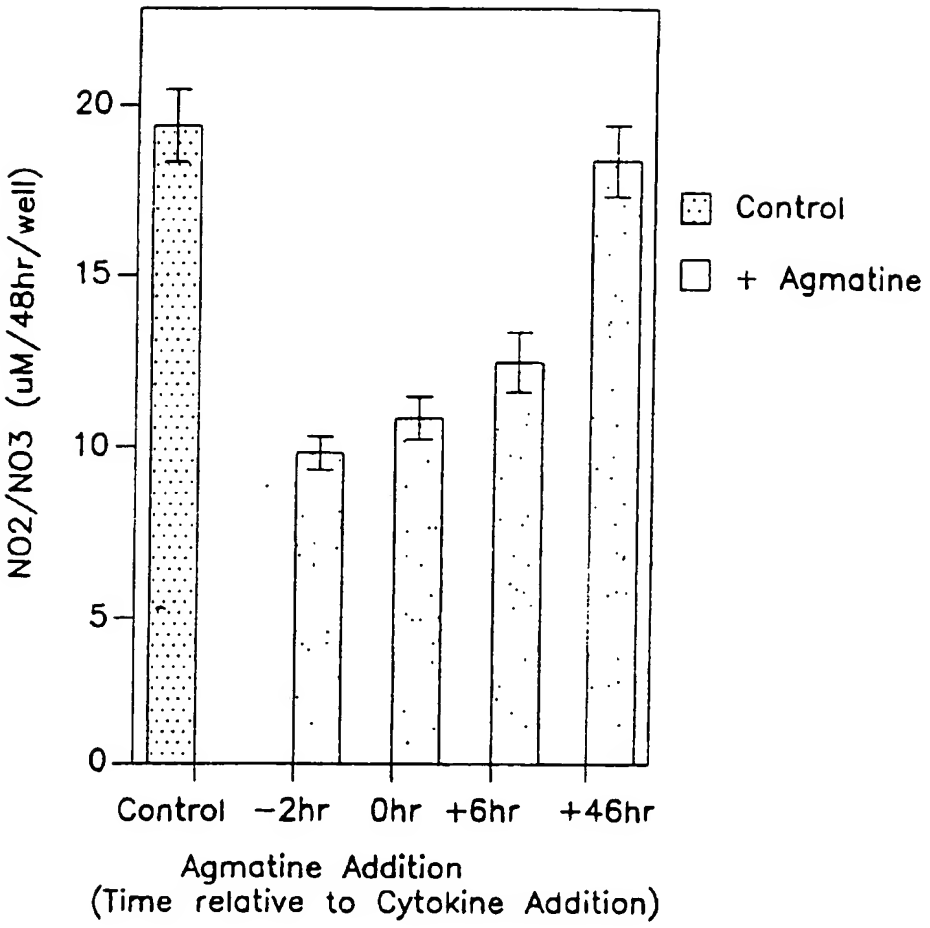

NO2/NO3 (uM/48hr/well)
20
15
10
5
0
Control
+ Agmatine
Control
−2hr
0hr
+6hr
+46hr
Agmatine Addition
(Time relative to Cytokine Addition)

FIG. 15

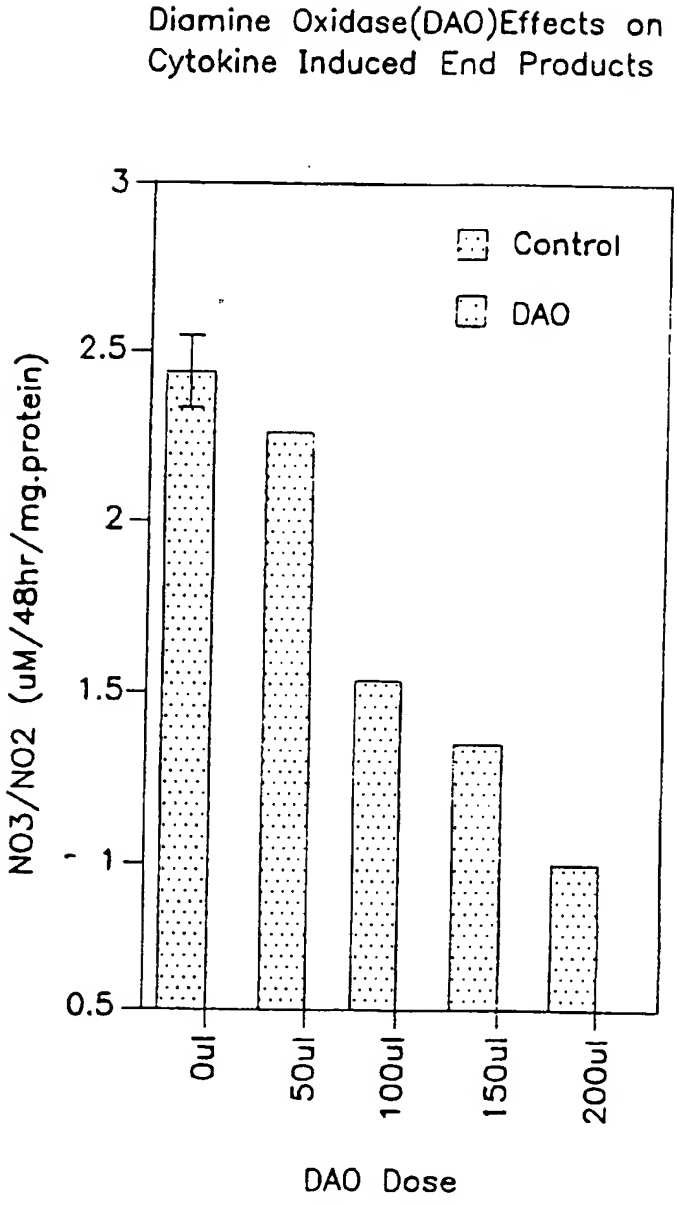

Diamine Oxidase(DAO)Effects on Cytokine Induced End Products
Control
DAO
NO3/NO2 (uM/48hr/mg.protein)
3
2.5
2
1.5
1
0.5
0ul
50ul
100ul
150ul
200ul
DAO Dose

FIG. 16A

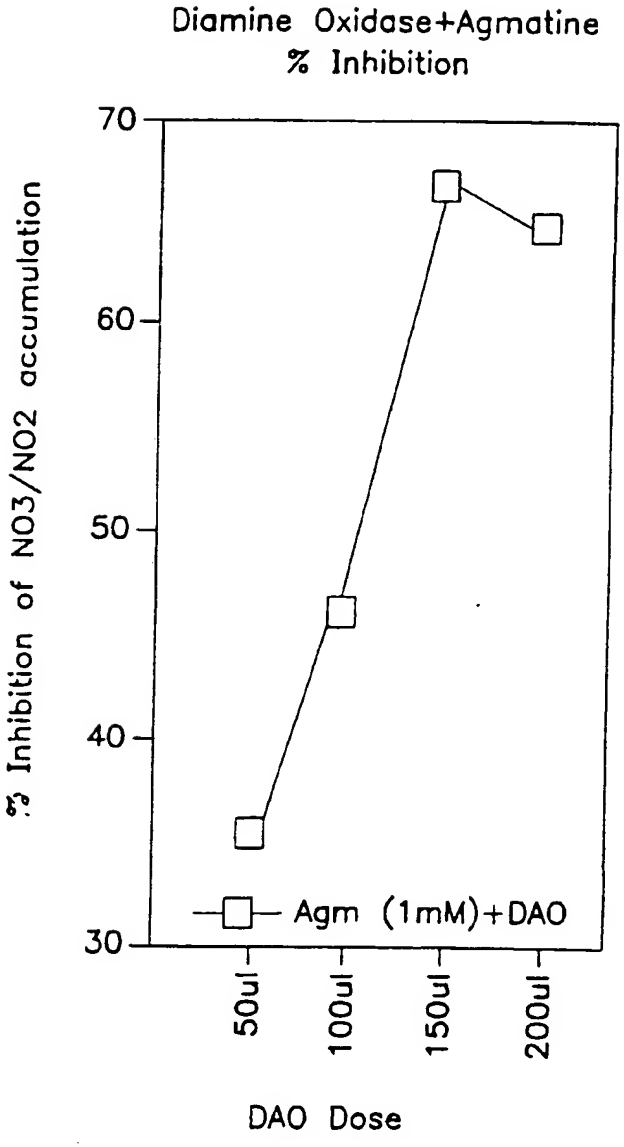

Diamine Oxidase+Agmatine
% Inhibition
% Inhibition of NO3/NO2 accumulation
70
60
50
40
30
Agm (1mM)+DAO
50ul
100ul
150ul
200ul
DAO Dose

FIG. 16B

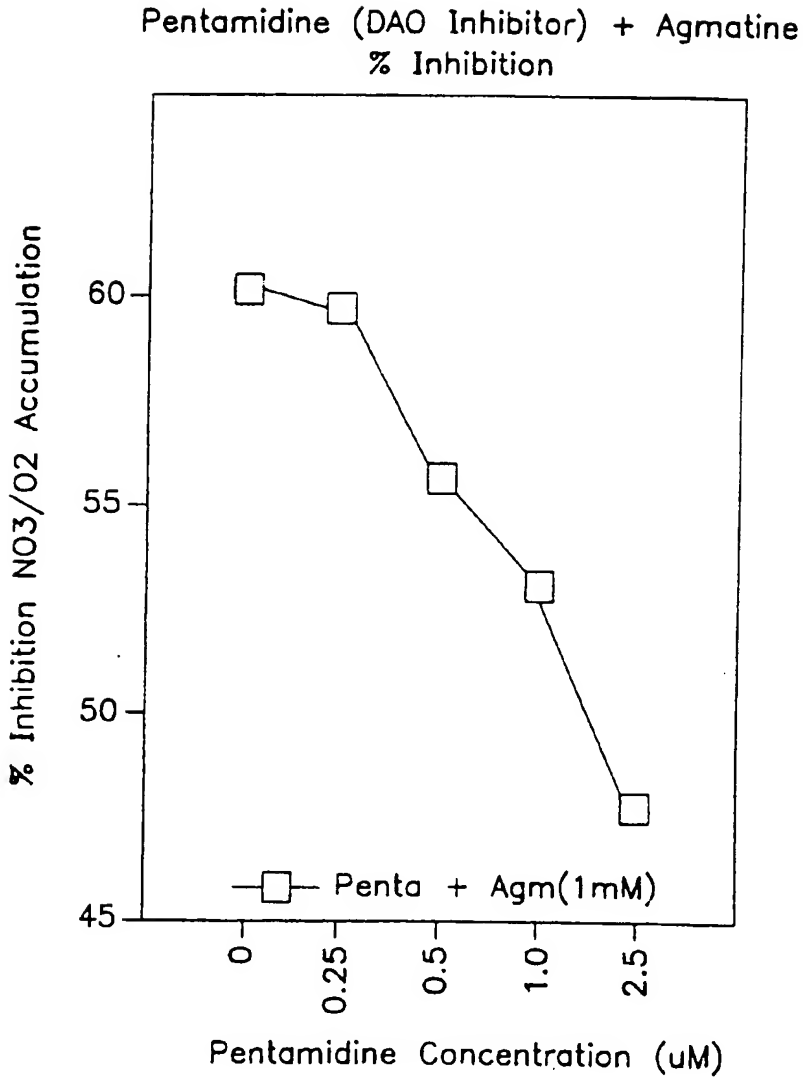

Pentamidine (DAO Inhibitor) + Agmatine
% Inhibition
% Inhibition NO3/O2 Accumulation
60
55
50
45
Penta + Agm(1mM)
0
0.25
0.5
1.0
2.5
Pentamidine Concentration (uM)

FIG. 17

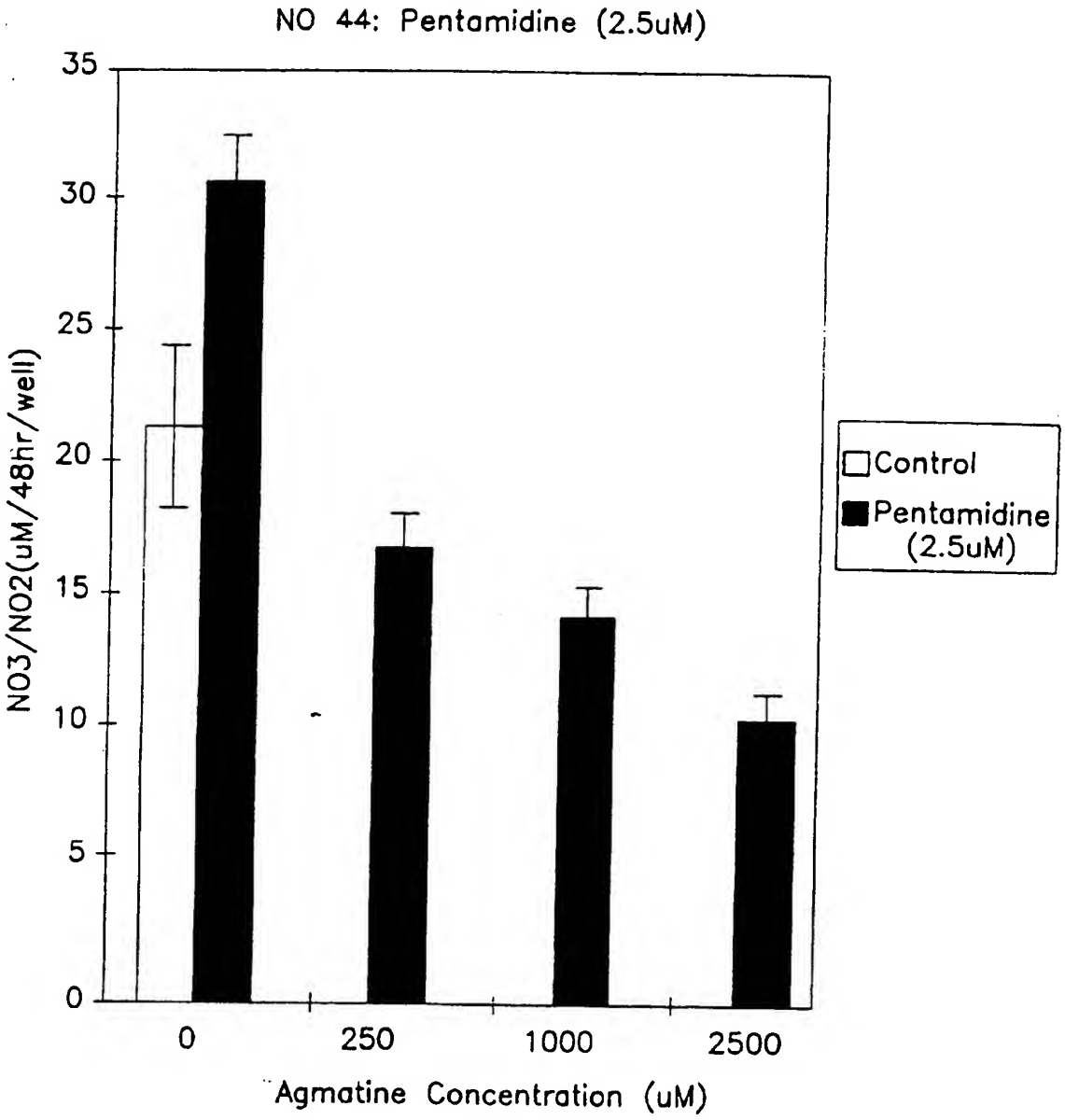

NO 44: Pentamidine (2.5uM)
NO3/NO2(uM/48hr/well)
35
30
25
20
15
10
5
0
0
250
1000
2500
Agmatine Concentration (uM)
Control
Pentamidine (2.5uM)

FIG. 18

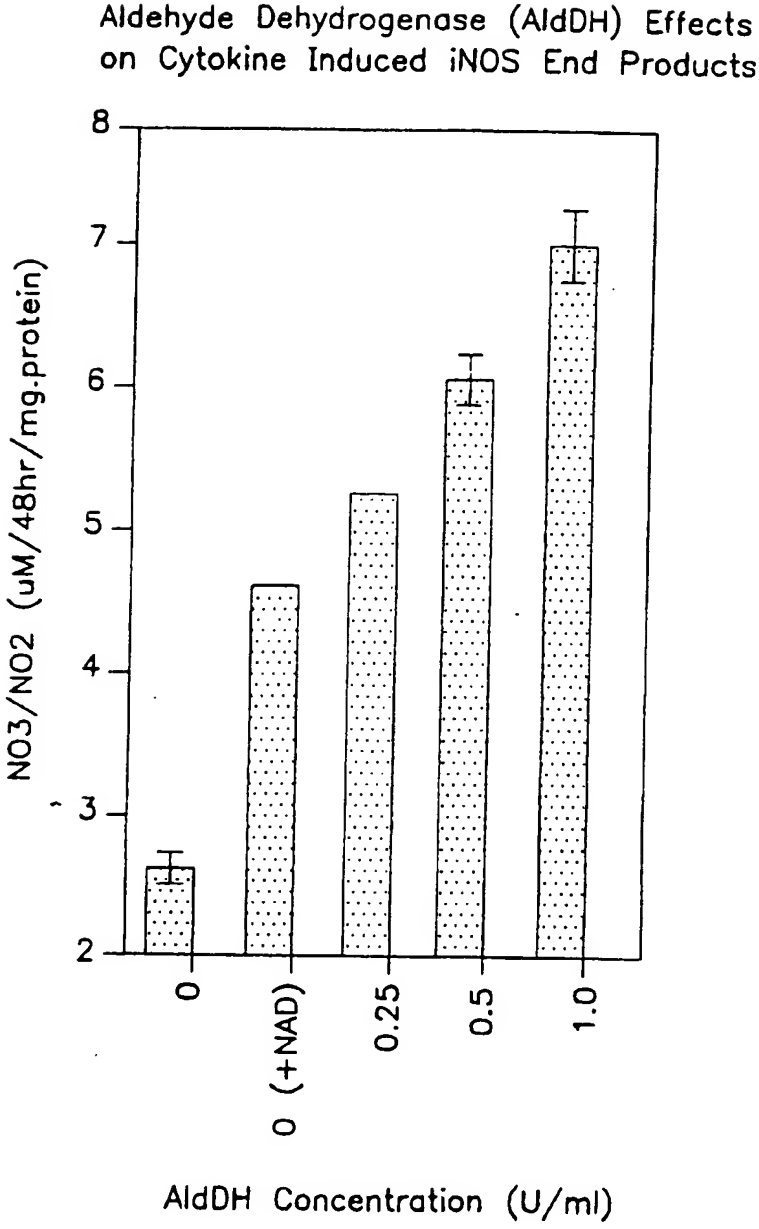

Aldehyde Dehydrogenase (AldDH) Effects
on Cytokine Induced iNOS End Products
NO3/NO2 (uM/48hr/mg.protein)
8
7
6
5
4
3
2
0
0 (+NAD)
0.25
0.5
1.0
AldDH Concentration (U/ml)

FIG. 19A

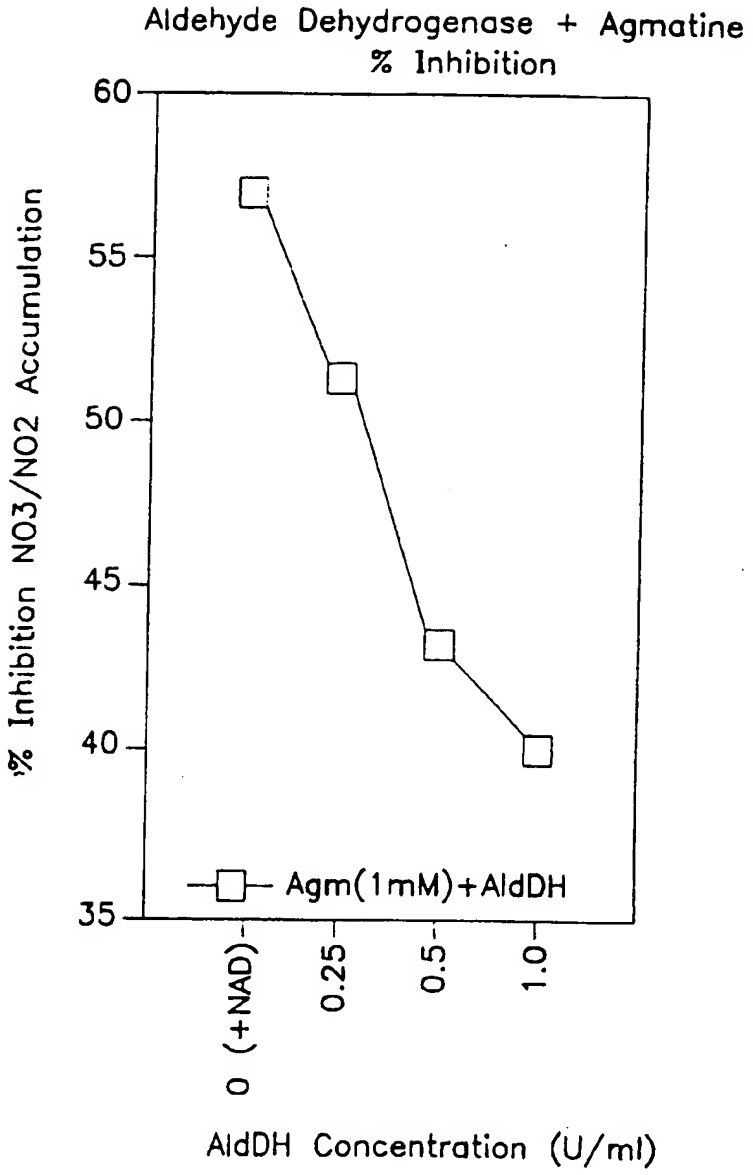

Aldehyde Dehydrogenase + Agmatine
% Inhibition
% Inhibition NO3/NO2 Accumulation
60
55
50
45
40
35
Agm(1mM)+AldDH
0 (+NAD)
0.25
0.5
1.0
AldDH Concentration (U/ml)

FIG. 19B

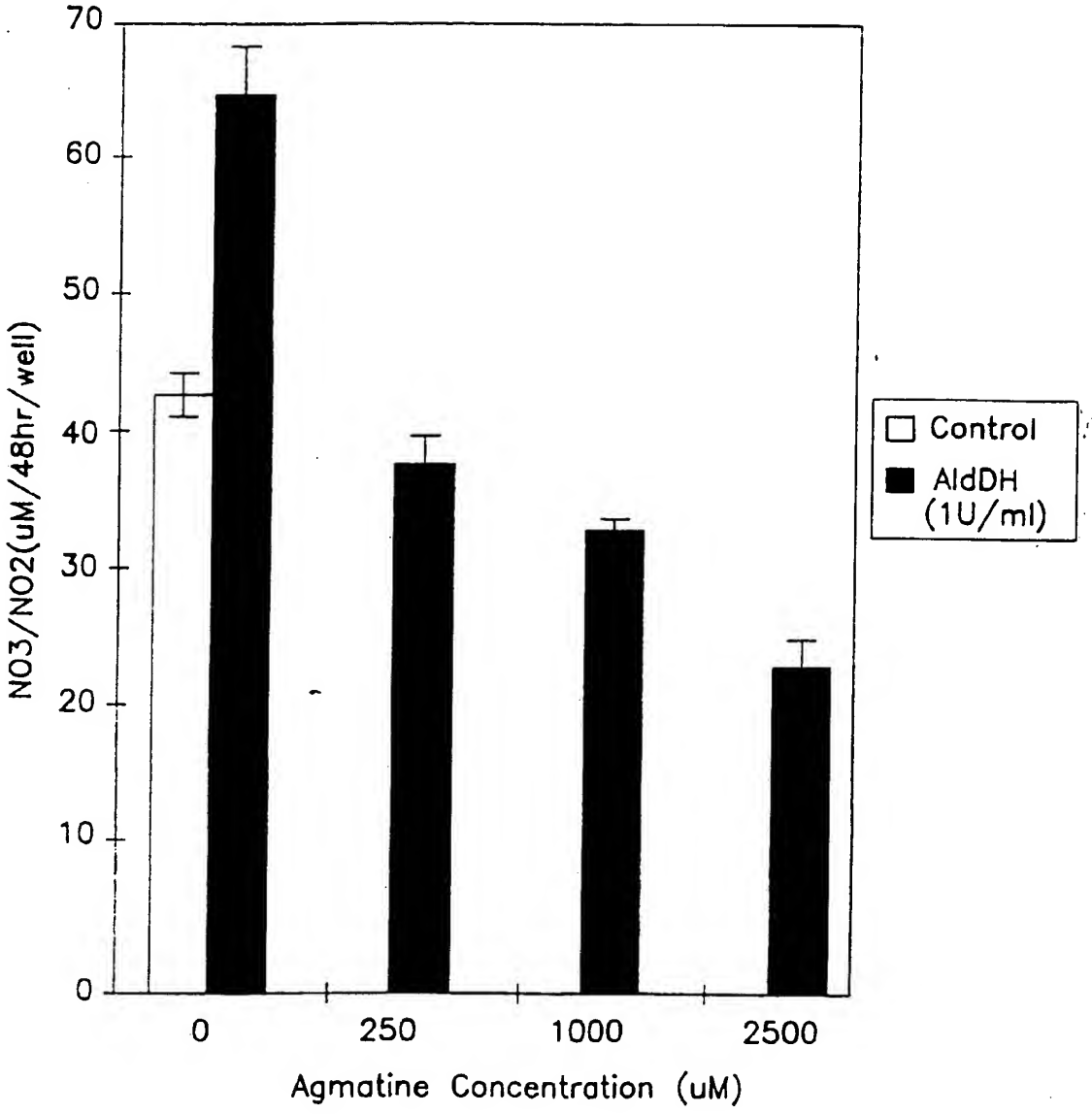

70
60
50
40
30
20
10
0
NO3/NO2(uM/48hr/well)
0
250
1000
2500
Agmatine Concentration (uM)
Control
AldDH
(1U/ml)

FIG. 20

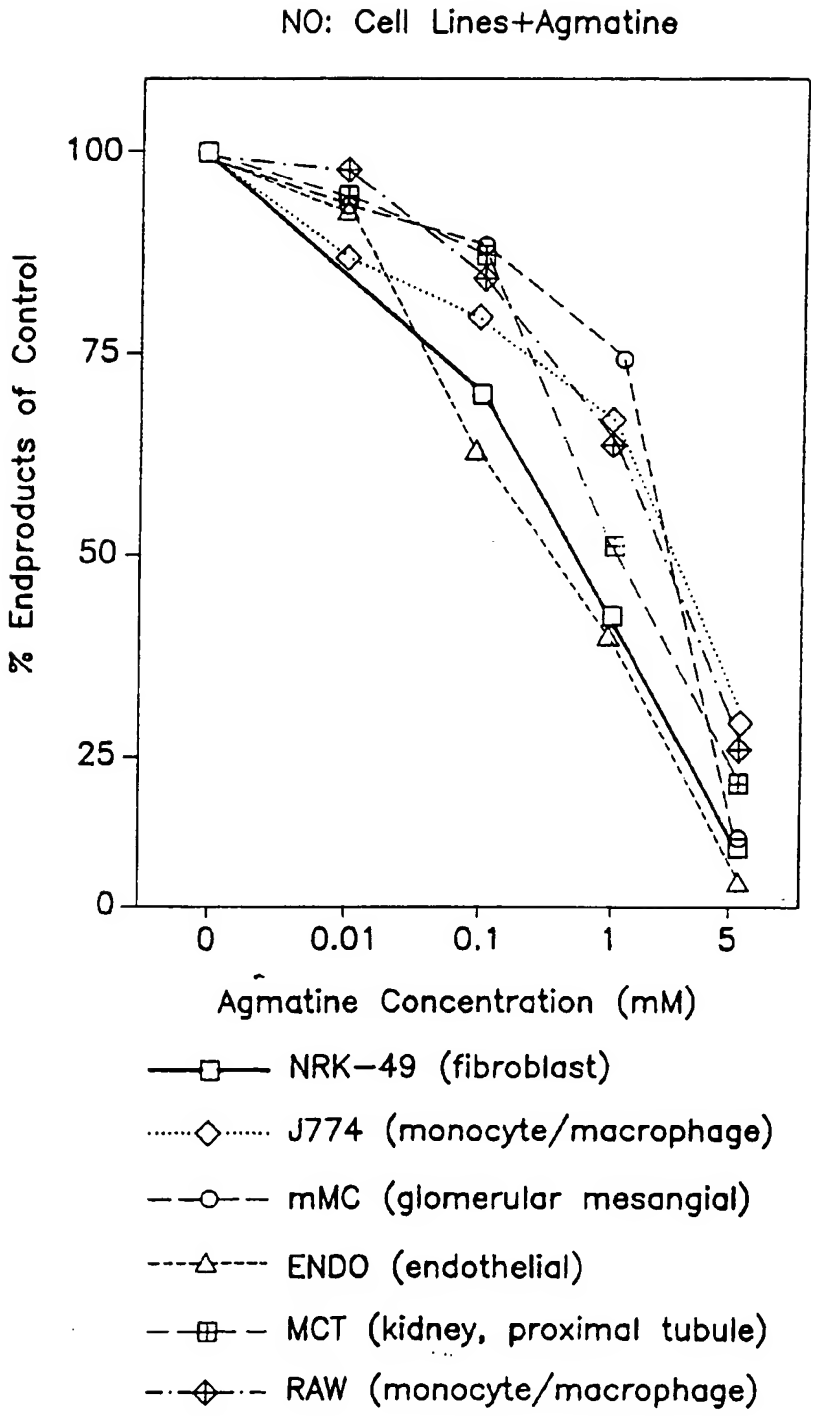

NO: Cell Lines+Agmatine
% Endproducts of Control
100
75
50
25
0
0
0.01
0.1
1
5
Agmatine Concentration (mM)
NRK-49 (fibroblast)
J774 (monocyte/macrophage)
mMC (glomerular mesangial)
ENDO (endothelial)
MCT (kidney, proximal tubule)
RAW (monocyte/macrophage)

FIG. 21

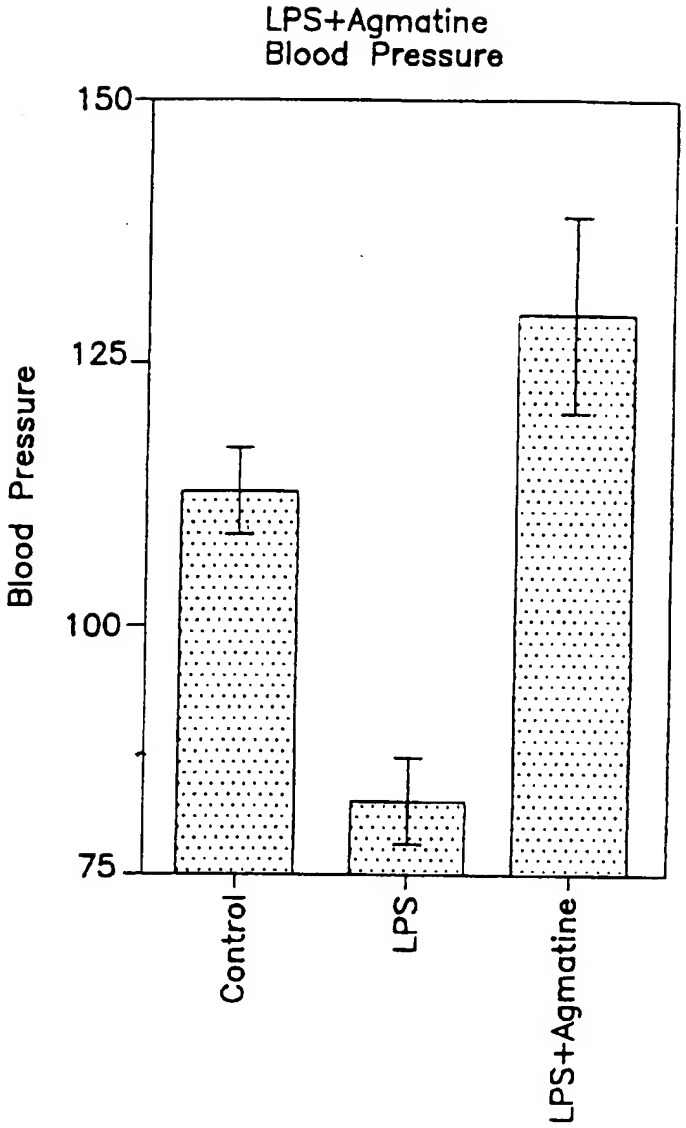

LPS+Agmatine
Blood Pressure
150
125
100
75
Blood Pressure
Control
LPS
LPS+Agmatine

FIG. 22A

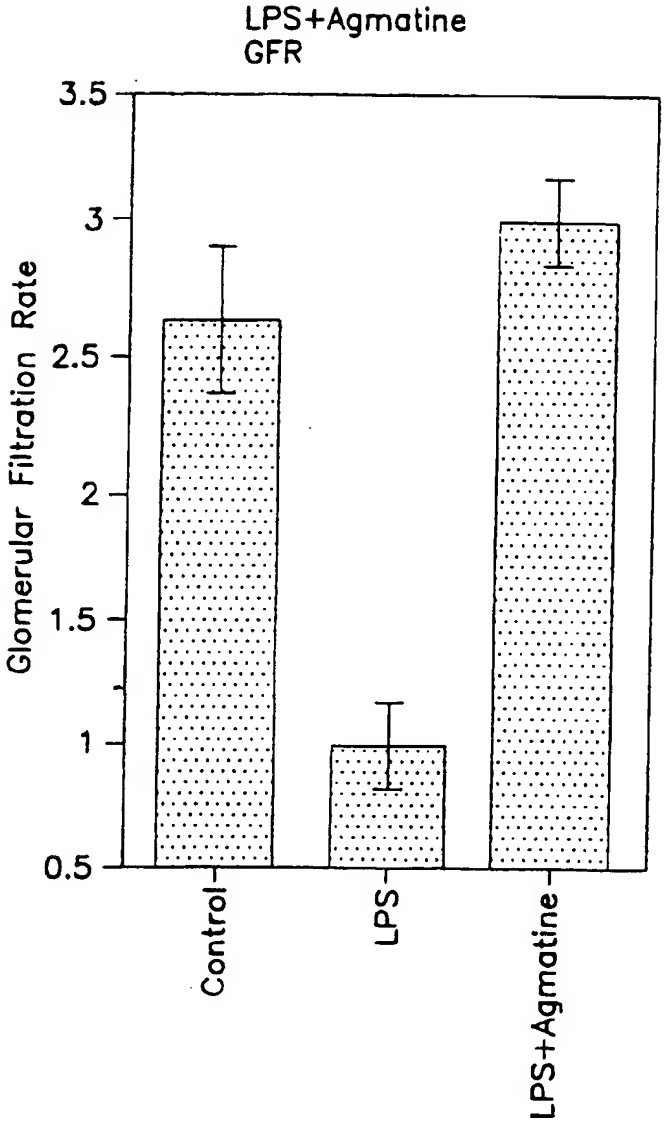

LPS+Agmatine
GFR
Glomerular Filtration Rate
3.5
3
2.5
2
1.5
1
0.5
Control
LPS
LPS+Agmatine

FIG. 22B

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US97/17424

A. CLASSIFICATION OF SUBJECT MATTER
IPC(6) :A61K 31/155, 31/415
US CL :514/634, 397
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 514/634, 397

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A,P	US 5,574,059 A (REGUNATHAN et al.) 12 November 1996, see entire document	1-34
A,E	US 5,677,349 A (GILAD et al.) 14 October 1997, see entire document.	1-34

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"B"	earlier document published on or after the international filing date	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
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Date of the actual completion of the international search	Date of mailing of the international search report
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